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(54) Title: LANTHANIDE CHELATE-TAGGED NUCLEIC ACID PROBES

(57) Abstract

Nucleic acid probes are provided which are chemically tagged with moieties which chelate the trivalent lanthanides Eu^{+3}, Tb^{+3} and Sm^{+3}. Also provided are methods of making said probes and methods of using the probes in hybridization assays. The probes of the invention are detected, preferably by time-resolved fluorometry, by means of the intense, long-lived fluorescence of Eu^{+3}, Tb^{+3} and Sm^{+3}, particularly in chelates with aromatic trifluoromethyl β-diketones, such as 2-naphthoyltrifluoroacetone, and synergistic bases, such as tri-n-octylphosphine oxide, when such chelates are in micelles, such as those formed in water with non-ionic detergents such as Triton X-100.

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LANTHANIDE CHELATE-TAGGED NUCLEIC ACID PROBES

TECHNICAL FIELD

The present invention relates to nucleic acid hybridization probes. More particularly, it relates to probes chemically labeled with chelates of fluorescent lanthanide ions and to processes for making and using such probes.

BACKGROUND OF THE INVENTION

The use of single-stranded DNA or RNA probes, to test for the presence of particular DNAs or RNAs and associated biological entities in samples of biological material, is well known. See e.g., Grunstein and Hogness, Proc. Nat'l. Acad. Sci. (US) 72, 3961-3965 (1975); Southern, J. Mol. Biol. 98, 503-505 (1975); Langer, et al., Proc. Nat'l. Acad. Sci. (US) 78, 6633-6637 (1981); Falkow and Moseley, U.S. Patent No. 4,358,535; Ward, et al., European Patent Application Publication No. 0 063 879; Englehardt, et al., European Patent Application Publication No. 0 097 373; Meinkoth and Wahl, Anal. Biochem., 138, 267-284 (1984).

Among areas in which such probes find application are testing of food and blood for contamination by pathogenic bacteria and viruses; diagnosis of fungal, bacterial and viral diseases by analysis of feces, blood or other body fluids; diagnosis of genetic disorders, and certain diseases such as cancers associated with a genetic abnormality in a population of cells, by analysis of cells for the absence of a gene or the presence of a defective gene; and karyotyping. See Klausner and Wilson, Biotechnology 1, 471-478 (1983); Englehardt, et al., supra; Ward, et al., supra; Falkow and Moseley, supra.

The principle which underlies the use of such probes is that a particular probe, under sufficiently stringent conditions, will, via hydrogen-bonding between complementary base moieties, selectively hybridize to (single-stranded) DNA or RNA which includes a sequence of nucleotides ("target sequence") that is complementary to a nucleotide sequence of the probe ("probing sequence" specific for the target sequence). Thus, if a biological entity (e.g., virus, microorganism, normal chromosome, mammalian chromosome bearing a defective gene) to be tested for has at least one DNA or RNA sequence uniquely associated with it in samples to be tested, the entity can be tested for using a nucleic acid probe.

A DNA or RNA associated with an entity to be tested for, and including a target sequence to which a nucleic acid probe hybridizes selectively in a hybridization assay, is called "target" DNA or RNA, respectively, of the probe.

A probe typically will have at least 8, and usually at least 12, ribonucleotides or 2'-deoxyribonucleotides in the probing sequence that are complementary to a target sequence in target DNA or RNA. Outside the probing sequences through which a probe complexes with its target nucleic acid, the probe may have virtually any number and type of bases, as long as the sequences including these additional bases do not cause significant hybridization with nucleic acid other than target nucleic acid under hybridizaton assay conditions. That is, a probe will be specific for its target DNA or RNA in hybridization assays.

To be useful in analyzing biological samples for the presence of target DNA or RNA, a polynucleotide probe must include a feature which will render detectable the duplex formed when the probe is hybridized to its complementary sequence in the target (single-stranded) DNA or RNA. Typically, such features in a probe include radioactive atoms, pyrimidine or purine bases chemically

modified to include moieties ("tag moieties") which can be detected by any of a number of techniques, or 5'-terminal phosphates similarly chemically modified.

For example, a probe may be made with ^{32}P-labeled nucleoside mono- or triphosphates; then the probe itself, as well as target DNA or RNA with the probe hybridized to it, can be detected by means of radiation from ^{32}P-decay.

Probes whose detectability is based on radioactive decay are unsuitable for many applications because of safety problems and licensing requirements associated with radioactive materials and because of degradation of the probes that occurs with radioactive decay during storage.

Alternatively, there are numerous examples of probes based on chemically modified nucleic acid. Some of these chemically labeled probes are detected by means of fluorescent, luminescent, or other emissive or absorptive properties of the tag moieties themselves or chemical entities which occur observably (i.e., significantly above background) in a detection system only if tag moiety (and, consequently probe) is present. See e.g., Ward, et al., supra; Englehardt, et al., supra; Klausner and Wilson, supra; Heller, et al., European Patent Application Publication No. 0 070 687.

With some of these chemically labeled probes, detection is, for example, by excitation of fluorescence from a fluorescent moiety, such as fluorescein, which is chemically linked directly to probe nucleic acid. With others of these probes, a ligand, such as biotinyl, is linked directly to probe nucleic acid and detection is by fluorescence excitation of a fluorescent moiety, such as fluorescein, conjugated to a molecule, such as streptavidin or anti-biotin antibody in the case of biotinyl ligand, which binds tightly to the ligand when combined with probe in a hybridization assay. With still

other of these probes, the ligand attached directly to probe is complexed with a "reporter group" which binds tightly to the ligand and which includes an active enzyme which catalyzes a reaction which produces a fluorescent, luminescent, or colorimetric product.

Probes detected by fluorescence employing techniques such as these, known heretofore, have a number of drawbacks. With probes detected by fluorescence, sensitivity (i.e., the minimum quantity of target nucleic acid that can be detected) is usually low, due to the intrinsic background fluorescence in hybridization assay systems; and this low sensitivity limits commercial applicability. Typically, 100 to 1,000 times more target is required for detection with a probe detected by means of fluorescence than with a ^{32}P-labeled probe.

Probes dependent on enzymatic reactions to generate fluorescent compounds suffer from a need for long incubation periods for acceptable sensitivity in most applications. Probes dependent on enzymes, antibodies or other complex biochemicals, such as streptavidin and biotin, for detectability suffer from the high cost of providing such materials with purity adequate for hybridization assays as well as the need for long incubation periods for detection.

The use of lanthanides as fluorescent tags in immunoassays has been reported. See Soini and Hemmila, U.S. Patent No. 4,374,120; Wieder and Wollenberg, U.S. Patent No. 4,352,751; Wieder (I), U.S. Patent No. 4,341,957; Wieder (II), U.S. Patent No. 4,058,732; Oy et al., European Patent Application No. 0 064 484; Hemmila et al., Anal. Biochem. 137, 334-335(1984); Halonen, et al., Current Topics in Microbiological Immunology 104, 133-146(1983); Soini and Kojola, Clin. Chem. 29, 65-68(1983).

Time-resolved fluorometry of rare-earth chelate fluorescent tags in immunoassays, and apparatus to carry out the procedure, have been reported. See Wieder (I) and Wieder (II), supra.

Time-resolved fluorometry of Eu^{+3} and Tb^{+3} chelates formed with a mixture of an aromatic trifluoromethyl ß-diketone and "synergistic" Lewis base has been reported in connection with immunoassays wherein antibody is labeled with a polyaminocarboxylate chelate of the lanthanide ion and wherein the ß-diketone/Lewis base chelate is formed by mixing the chelate-labeled antibody with a solution, buffered to acid pH, of a detergent and the ß-diketone and "synergistic" Lewis base. Oy, supra; Hemilla (1984), supra; and Halonen et al. (1983), supra. Several features of this technique provide a better signal to noise ratio (and, consequently, greater sensitivity) than other fluorescence-based detection techniques. These features include: (a) the use of time-resolved fluorescence (i.e., time-resolved fluorometry) which allows the collection of fluorescence emission signal from a sample in discrete time intervals after fluorescence excitation, so that the intrinsic, relatively intense but relatively short-lived background fluorescence of biological materials (e.g., protein, nucleic acid) can decay to near zero before measurement of the long-lived fluorescence of lanthanide in chelates begins; (b) some trivalent lanthanide ions, especially Eu(III), have other fluorescent properties which further accentuate the signal to noise ratio, such as a broad excitation bandwidth, narrow emission bandwidth, and a large Stoke's shift (difference between frequencies of excitation and emission maxima); and (c) chelating with the aromatic trifluoromethyl ß-diketone and synergistic Lewis base and sequestering the chelate in a micelle away from water (which tends to quench fluorescence emission) enhances fluorescence intensity of Eu^{+3} by up to six orders of magnitude over the intensity in an aqueous environment without the ß-diketone or synergistic base. In an aqueous solution, as little as about 10 attomoles (10×10^{-18} moles) of Eu^{+3} can be determined with

time-resolved fluorometry of chelates of the Eu^{+3} formed with aromatic, trifluoromethyl ß-diketone and synergistic Lewis base which have been sequestered in micelles.

Fluorescence of trivalent lanthanide ions, particularly Eu^{+3} and Tb^{+3}, bound directly to nucleic acids has been employed to detect the presence of nucleic acids in biological specimens and to study the structure and conformation of nucleic acids. See Richardson, Chem. Rev. 82, 541-552 (1982).

Chu and Orgel, Proc. Natl. Acad. Sci. (U.S.A.) 82, 963-967 (1985), and Dreyer and Dervan, Proc. Natl. Acad. Sci. (U.S.A.) 82, 968-972 (1985), report oligonucleotides covalently linked to chelates of ferrous ion with ethylene diaminetetraacetic acid and diethylenetriaminepentaacetic acid. In aqueous solution, hydroxyl radicals produced by the ferrous ion in the presence of O_2 cleave oligonucleotides.

Hemmila et al., supra, and Leung and Meares, Biochem. and Biophys. Res. Commun. 75, 149-155 (1977) have employed 1-(p-diazo-phenyl)EDTA to non-specifically label proteins with EDTA chelates of lanthanide ions. Forster et al., Nucl. Acids Res. 13, 745-761 (1985) describe the use of a photoactivatable, 4-azido-2-nitrophenyl derivative of biotin to non-specifically label DNA with biotin.

It has not heretofore been appreciated that nucleic acid hybridization probes can be labeled with tag moieties that chelate lanthanide ions, especially Eu(III), Tb(III), and Sm(III), and that thereby the fluorescent properties, as well as ease of use and low cost, of chelates of such ions can be exploited to overcome the various problems associated with other, particularly fluorescence-based, probe detection systems and provide probes of extraordinary sensitivity.

SUMMARY OF THE INVENTION

We have discovered nucleic acid hybridization probes tagged with chelating agents of trivalent europium, terbium and samarium. More specifically, we have discovered nucleic acid probes, DNA or RNA, labeled with polyaminocarboxylate derivatives that form chelates with high association constants with Eu(III), Tb(III), and Sm(III) in aqueous solution.

The probes of the invention are complexed with Eu^{+3}, Tb^{+3} or Sm^{+3} and are detected by means of the intense fluorescence of these ions, particularly in chelates formed with aromatic trifluoromethyl β-diketones and synergistic Lewis bases that can readily be prepared in hybridization assay systems with probes of the invention.

Our invention also entails methods of making, and intermediates for use in making, probes of the invention and methods of using the probes in nucleic acid hybridization assays.

The probes of the invention are substantially improved over known probes, including in particular those detected by fluorescence. Detection of probes of the invention involves only inexpensive, stable, readily available chemicals and no enzymes, proteins or other complex and costly materials. Further, detection of probes of the invention is quite simple, involving no complex biochemical steps. The probes of the invention involve no radioactive substances and none of the problems attendant with probes labeled or detected with such substances. Particularly when detection is by time-resolved fluorescence with chelates formed with a β-diketone and a synergistic Lewis base in micelles, the sensitivity of probes of the invention is greater than that of known chemically tagged probes and is comparable to or greater than that of probes labeled radioactively to high specific activity.

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention is a nucleic acid probe, DNA or RNA, which comprises a group of formula $-F_1L_1F_2R_1$, wherein $-F_1-$ and $-F_2-$ are functional groups at the termini of a linking moiety, $-F_1L_1F_2-$, separated by a spacer group, $-L_1-$, wherein $-R_1$ is a tag moiety that is a chelator of europium (III), terbium (III) or samarium (III), and wherein the group is bonded through $-F_1-$ to a nucleoside base of the probe, to a 5'-terminal nucleotide of the probe through the 5'-carbon of said 5'-terminal nucleotide, or to a 3'-terminal nucleotide of the probe through the 3'-carbon of said 3'-terminal nucleotide. In probes of the invention wherein a tag moiety R_1 is linked to the 5'-terminal nucleotide through the 5'-carbon thereof, the group bonded to said 5'-carbon can be of formula $-F_2R_1$.

The 5'-carbon of a 5'-terminal nucleotide of a polynucleotide is referred to herein as the "5'-terminal carbon." Similarly, the 3'-carbon of a 3'-terminal nucleotide of a polynucleotide is referred to herein as the "3'-terminal carbon."

Reference herein to "polynucleotide" means any polymer of ribonucleotides or 2'-deoxyribonucleotides joined by 5'-3'- phosphodiester bonds and includes oligonucleotides as well as longer polymers. Usually all of the nucleotides of a polynucleotide will be either ribonucleotides or 2'-deoxyribonucleotides. However, in some cases, described below, a polynucleotide which otherwise consists of 2'-deoxyribonucleotides might terminate with a ribonucleotide followed immediately, at the 3'-terminus, with a 2'-deoxyribonucleotide or a polynucleotide which otherwise consists of ribonucleotides might terminate with a 2'-deoxyribonucleotide.

When the group $-F_1L_1F_2R_1$ is bonded to a 5'-terminal carbon of a probe of the invention,

$-F_1L_1-$ is typically $-O\overset{\overset{O}{\|}}{\underset{\underset{O}{|}}{P}}(NH)L_1-$, $-NHL_1-$, $-NH(C{=}S)NHL_1-$,

$-O\overset{\overset{O}{\|}}{\underset{\underset{O}{|}}{P}}O(L_6)SSL_1-$, $-NH(C{=}O)NHL_1-$, or $-O\overset{\overset{O}{\|}}{\underset{\underset{O}{|}}{P}}S(CH_2)(CO)L_1-$,

wherein L_6 is alkyl of 3 to 20 carbon atoms; L_1 is typically an alkyl group of 2 to 20 carbon atoms or an alkyl group of 2 to 18 carbon atoms interspersed with an amide linkage (i.e., of formula $-L_{101}(NH)(CO)L_{102}-$ or $-L_{101}(CO)(NH)L_{102}-$ wherein L_{101} is alkyl of 2 to 17 carbon atoms and bonded to F_1, L_{102} is alkyl of 1 to 17 carbon atoms and L_{101} and L_{102} together have no more than 18 carbon atoms; and $-F_2R_1$ is typically $-NHR_1$, $-NH(C{=}O)NHR_1$, $-NH(C{=}S)NHR_1$, or $-S(CH_2)(CO)NHR_1$. The preferred linking moieties bonded to the 5'-terminal carbon of a probe of the invention are $-OPO_2NH(CH_2)_nNH-$, wherein n is 2 to 8.

When the group $-F_2R_1$ is bonded to a 5'-terminal carbon of a probe of the invention, $-F_2-$ is typically $-NH-$, $-NH(C{=}S)NH-$ or $-NH(C{=}O)NH-$, preferably $-NH-$.

When the group $-F_1L_1F_2R_1$ is bonded to a 3'-terminal carbon of a probe of the invention,

$-F_1L_1-$ is typically $-O\overset{\overset{O}{\|}}{\underset{\underset{O}{|}}{P}}(NH)L_1-$ or $-O\overset{\overset{O}{\|}}{\underset{\underset{O}{|}}{P}}SCH_2(CO)L_1-$,

L_1 is typically alkyl of 2 to 20 carbon atoms or $-L_{101}(CO)(NH)L_{102}-$ or $-L_{101}(NH)(CO)L_{102}-$, wherein L_{101} is alkyl of 2 to 17 carbon atoms, L_{102} is alkyl of 1 to 17 carbon atoms, and L_{101} and L_{102} together have no more than 18 carbon atoms; and $-F_2R_1$ is typically $-NHR_1$, $-NH(C{=}S)NHR_1$, $-NH(C{=}O)NHR_1$, or $-S(CH_2)(CO)NHR_1$. The preferred linking moieties

bonded to the 3'-terminal carbon are $-OPO_2NH(CH_2)_nNH-$, wherein n is 2 to 8.

The group $-O\underset{\underset{O}{|}}{\overset{\overset{O}{\|}}{P}}(NH)-$ is represented herein as "$-OPO_2NH-$" or "$-OPO_2(NH)-$". The group $-O\underset{\underset{O}{|}}{\overset{\overset{O}{\|}}{P}}O(L_6)$ is is represented herein as "$-OPO_3(L_6)-$" or "$-OPO_3L_6-$". The group $-O\underset{\underset{O}{|}}{\overset{\overset{O}{\|}}{P}}S(CH_2)-$ is represented herein as "OPO_2SCH_2" or $-OPO_2S(CH_2)-$".

If the probe has a group of formula $-OPO_2SCH_2(CO)L_1F_2R_1$ bonded to the 3'-terminal carbon, the 3'-terminal nucleotide of the probe will be a 2'-deoxyribonucleotide and the next nucleotide in the 5'-direction from said 3'-terminal nucleotide will be a ribonucleotide, regardless of whether the remainder of the probe is 2'-deoxyribonucleotides or ribonucleotides.

When the group $-F_1L_1F_2R_1$ is bonded to a nucleoside base of the probe, it will preferably be bonded to the 5-position of a uracil moiety, although it can be bonded to other positions, including the 5-position or N^4-nitrogen of a cytosine moiety and the 8-position of a guanine or adenine moiety.

When the group $-F_1L_1F_2R_1$ is bonded to carbon-5 of a pyrimidine moiety, $-F_1L_1-$ will typically be selected from $-CH=CHL_1-$, $-CH=CH(CO)(NH)L_1-$, $-(CH_2)_2(CO)(NH)L_1-$, and $-CH=CHCH_2(NH)(CO)_zL_1-$, wherein z is 0 or 1; wherein, when $-F_1-$ is $-CH=CH-$, $-CH=CH(CO)(NH)-$, $-(CH_2)_2(CO)(NH)L_1-$, or terminated with a carbonyl group, $-L_1-$ will typically be n-alkyl of 1 to 20 carbon atoms, $-L_{101}(NH)(CO)L_{102}-$ or $-L_{101}(CO)(NH)L_{102}-$, wherein $-L_{101}-$ is bonded to $-F_1-$ and is n-alkyl of 1 to 17 carbon atoms, $-L_{102}-$ is alkyl of 1 to 17 carbon

atoms and L_{101} and L_{102} together have no more than 18 carbon atoms; wherein, when $-F_1-$ is terminated with an amino group (i.e., z is 0), $-L_1-$ will typically be $-CH_2(CHOH)CH_2O(CH_2)_yOCH_2(CHOH)CH_2-$, wherein y is 2 to 20 (preferably 4); and wherein $-F_2R_1$ will typically be $-NHR_1$, $-NH(C{=}S)NHR_1$ or $-NH(C{=}O)NHR_1$. Most preferably, the linker moiety $-F_1L_1F_2-$ bonded to the carbon-5 of a pyrimidine in probes of the invention is of formula $-CH{=}CH{-}CH_2{-}NH-$.

When the group $-F_1L_1F_2R_1$ is bonded to the N^4-nitrogen of a cytosine moiety, $-F_1L_1-$ will typically be selected from $-N{=}C(R_{22})L_1-$, $-NHL_1-$, $-NH(C{=}O)NHL_1-$, or $-NH(C{=}S)NHL_1$, wherein R_{22} is hydrogen or alkyl of 1 to 4 carbon atoms; $-L_1-$ will typically be selected from alkyl of 2 to 20 carbon atoms, preferably $-(CH_2)_r-$, wherein r is 2 to 8; and $-F_2-$ is typically $-NH-$, $-NH(C{=}O)NH-$ or $-NH(C{=}S)NH-$. In the group $-F_1L_1F_2R_1$ bonded to an N^4-nitrogen of cytosines in probes of the invention, $-F_1L_1F_2-$ is preferably $-N{=}CH(CH_2)_rNH-$.

When the group $-F_1L_1F_2R_1$ is bonded to carbon-8 of a purine moiety, $-F_1L_1-$ is typically O, S or $-NH-$; $-L_1-$ is typically n-alkyl of 1 to 20 carbon atoms, $-L_{105}(NH)(CO)L_{106}-$ or $-L_{105}(CO)(NH)L_{106}-$, wherein $-L_{105}$ is n-alkyl of 1 to 17 carbon atoms and is bonded to F_1, $-L_{106}$ is alkyl of 1 to 17 carbon atoms, and L_{105} and L_{106} together have no more than 18 carbon atoms; and $-F_2-$ is typically selected from $-NH-$, $-NH(C{=}O)NH-$ and $-NH(C{=}S){-}NH-$. Most preferably, the linker moiety $-F_1L_1F_2-$ bonded to the carbon-8 of a purine in probes of the invention is of formula $-NH(CH_2)_pNH-$, wherein p is 2 to 8.

The tag moiety-chelating agent $-R_1$ will preferably have a dissociation constant with Eu^{+3}, Tb^{+3} and Sm^{+3} in aqueous solution at 25°C between pH 5 and pH 9 that is less than 10^{-17}M.

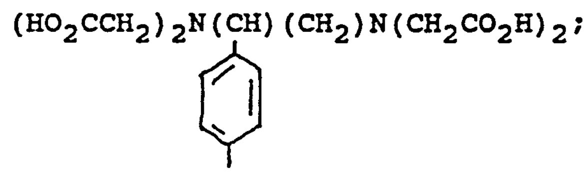
The preferred groups, R_1, for probes of the invention are EDTAyl, of formula:

$$\begin{array}{l} -(CO)CH_2 \\ \qquad\qquad \diagdown \\ \qquad\qquad\quad N(CH_2)_2N(CH_2CO_2H)_2; \\ \qquad\qquad \diagup \\ (HO_2C)CH_2 \end{array}$$

DTPAyl, of formula:

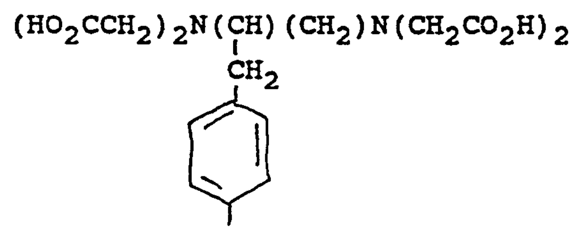
$$\begin{array}{l} -(CO)CH_2 \qquad\qquad\quad CH_2CO_2H \\ \qquad\qquad \diagdown \qquad\qquad\quad | \\ \qquad\qquad\quad N(CH_2)_2N(CH_2)_2N(CH_2CO_2H)_2; \text{ and} \\ \qquad\qquad \diagup \\ (HO_2C)CH_2 \end{array}$$

p-EDTA-phenyl, of formula:



and

p-EDTA-benzyl, of formula:



EDTA is an abbreviation for ethylenediaminetetraacetic acid.

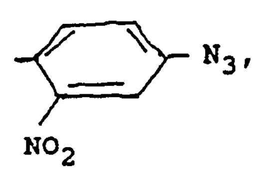
DTPA is an abbreviation for diethylenetriaminepentaacetic acid.

Included in the probes of the invention are those wherein the tag moieties, R_1, are complexed with Eu^{+3}, Tb^{+3}, or Sm^{+3}. That is, in the probes of the invention, tag moiety R_1 is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

Reference herein to a chelating group (e.g., DTPAyl, EDTAyl, p-EDTA-phenyl or p-EDTA-benzyl), or a compound of which the group is a part, being

"optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}" means that either the group chelates one of these lanthanide III ions or the group does not chelate any of the three lanthanide III ions. If the chelating group does not chelate Eu^{+3}, Tb^{+3} or Sm^{+3}, it might nonetheless, as the skilled will understand, be complexed with other metal ions, that might be present in solution with the chelating group, such as, for example, Na^{+} or K^{+} from buffers in the solution or magnesium, manganese, cobalt or other metal ions present in connection with enzymes.

In another of its aspects, the present invention includes a DNA or RNA probe which is made by a process which comprises reacting 1-(p-diazo-phenyl)EDTA, optionally (and preferably) complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, or a phenyl-azide-derivatized EDTA or DTPA of formula $(R_{263})NH(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}NH(R_{261})$, wherein R_{261} is EDTAyl or DTPAyl and is optionally (and preferably) complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, with a DNA or RNA with the sequence of the probe. In the phenyl-azide-derivatized EDTA or DTPA of formula $(R_{263})NH(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}NH(R_{261})$, R_{263} is of formula



R_{264} is H or n-alkyl of 1 to 3 carbon atoms, aa is 1 to 6, bb is 1 to 6 and cc is 0 or 1.

The phenyl-azide-derivatized EDTAs or DTPAs of formula $(R_{263})(NH)(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}NH(R_{261})$, wherein R_{261} is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3} and wherein R_{261}, R_{263}, R_{264}, aa, bb and cc are as defined in the preceding paragraph, are novel and also an aspect of the present invention.

Reference herein to "phenyl azide-derivatized EDTA or DTPA" is, unless otherwise specifically qualified, to compounds of formula $(R_{263})(NH)(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}NH(R_{261})$ as defined above in this paragraph.

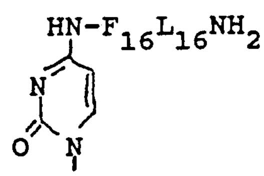
The present invention entails also duplexes between probes of the invention and their respective target DNA's or RNA's.

In another aspect, the present invention entails methods of making probes of the invention.

Methods of making a polynucleotide (DNA or RNA) which comprises a pyrimidine with a moiety of formula $-F_{15}L_{15}NH_2$ bonded to the carbon-5 position, wherein $-F_{15}-$ is selected from $-CH{=}CH-$, $-(CH_2)_2(CO)(NH)-$, $-CH{=}CHCH_2NH(CO)_x-$, and $-CH{=}CH(CO)(NH)-$; wherein the group $-CH{=}$ or $-(CH_2)_2$ is bonded to the carbon-5; wherein x is 0 or 1; wherein, when $-F_{15}$ is $-CH{=}CH-$, $-(CH_2)_2(CO)(NH)-$, $-CH{=}CH(CO)(NH)-$ or a group terminated with a carbonyl group, $-L_{15}-$ is n-alkyl of 1 to 20 carbon atoms, $-L_{151}(NH)(CO)L_{152}-$ or $-L_{151}(CO)(NH)L_{152}-$, wherein $-L_{151}$ is bonded to F_{15} and is n-alkyl of 1 to 17 carbon atoms, L_{152} is alkyl of 1 to 17 carbon atoms and L_{151} and L_{152} together have no more than 18 carbon atoms; and wherein, when $-F_{15}-$ is terminated with an amino group, $-L_{15}-$ is $-CH_2(CHOH)CH_2O(CH_2)_wOCH_2(CHOH)CH_2-$, wherein w is 2 to 20, are known in the art. See, e.g., for enzymatic methods, Langer et al., supra; Ward et al., supra; Englehardt et al., supra; and Brakel et al., European Patent Application Publication No. 0 122 614. See, e.g., for solid phase stepwise methods, Ruth, Published Patent Cooperation Treaty Application No. WO 84/03285. For synthesis of pyrimidine -2'-deoxynucleosides wherein the 5-position of the base is bonded to a group of formula $-(CH_2)_2(CO)(NH)L_{15}NH_2$ and which can be employed in solid phase stepwise methods of synthesizing polynucleotides, see Dreyer and Dervan, supra.

Methods described by Dreyer and Dervan, supra, can also be employed to make, by solid-phase phosphoramidite chemistry, a precursor of a probe of the invention wherein, at one or more pyrimidine nucleotides in the sequence, a group of formula $O(CH_2)_2(CO)(NH)L_{15}(NH)$(EDTAyl-triester) is bonded to carbon-5 of the pyrimidine moiety. The polynucleotide with free EDTAyl group(s) linked to pyrimidines is obtained by treating the polynucleotide (linked to EDTAyl-triester groups), after detachment from the solid phase, with glacial acetic acid and then isolating chromatographically and electrophoretically. By treating the purified, EDTAyl-linked polynucleotide by the standard probe chelation procedure described below, a probe of the invention with EDTAyl linked by the group of formula $-(CH_2)_2(CO)(NH)L_{15}(NH)-$ to the 5'-carbon of pyrimidines and complexed with Eu^{+3}, Tb^{+3} or Sm^{+3} is obtained. In these probes, L_{15} is preferably n-alkyl of 2 to 8 carbons and the EDTAyl is preferably linked to uracil moieties.

A polynucleotide (DNA or RNA) wherein one or more of the cytosines are modified to a moiety of formula



wherein $-F_{16}-$ is -N=CH-, -NH, NH(C=S)NH, or NH(C=O)NH-; and wherein $-L_{16}-$ is alkyl of 2 to 20 carbon atoms, can be prepared following the teachings of Musso et al., U.S. Patent Application Serial No. 748,499, filed June 25, 1985, assigned to the assignee of the present application and incorporated herein by reference. Generally, a nucleic acid with the sequence of the probe is reacted with hydrazine in the presence of bisulfite near neutral pH to convert a fraction of the amino groups bonded to carbon-4 of cytosines to hydrazine groups, the nucleic acid with the $-NH-NH_2$ groups bonded to carbon-4 of cytosines is then reacted with a compound of formula

$(OHC)(L_{16})F_{17}$, $O{=}C{=}N(L_{16})F_{17}$ or $S{=}C{=}N(L_{16})F_{17}$, wherein F_{17} is a suitably protected amino group, then deprotection is carried out to yield an $-NH_2$ group from F_{17} in groups bonded to the N^4-nitrogens, and finally, if the hydrazone linkage $-N{=}CH{-}L_{16}-$ resulting from reaction with the aldehyde $(OHC)L_{16}F_{17}$ is to be converted to the hydrazide linkage, $-NH{-}CH_2{-}L_{16}$, reduction is carried out.

A polynucleotide (DNA or RNA) which comprises a purine with a moiety of formula $-F_{18}L_{18}NH_2$ bonded to the carbon-8 position, wherein $-F_{18}-$ is O, S or NH and L_{18} is n-alkyl of 1 to 20 carbon atoms, $-L_{181}(NH)(CO)L_{182}-$ or $-L_{181}(CO)(NH)L_{182}-$, wherein $-L_{181}-$ is n-alkyl of 1 to 17 carbon atoms and is bonded to F_{18}, $-L_{182}-$ is alkyl of 1 to 17 carbon atoms, and L_{181} and L_{182} together have no more than 18 carbon atoms, can be prepared by solid-phase, stepwise methods known in the art. See Ruth, supra.

A polynucleotide which has the sequence of a probe and which comprises a pyrimidine moiety with a group of formula $-F_{15}L_{15}NH_2$ bonded to the carbon-5, a cytosine moiety with a group of formula $-F_{16}L_{16}NH_2$ bonded to the N^4-nitrogen, or a purine moiety with a group of formula $-F_{18}L_{18}NH_2$ bonded to the carbon-8, wherein $-F_{15}$, F_{16}, F_{18}, L_{15}, L_{16} and L_{18} are as defined above, upon reaction with a suitable compound which includes tag moiety-chelator R_1, and which is suitable for nucleophilic attack by the amino group at the terminus of the $-F_{15}L_{15}NH_2$, $-F_{16}L_{16}NH_2$ or $-F_{18}L_{18}NH_2$ group will yield probe of the invention, wherein at least a fraction of the group or groups of formula $-F_{15}L_{15}NH_2$, $-F_{16}L_{16}NH_2$ or $-F_{18}L_{18}NH_2$ on the polynucleotide are replaced with a group of formula $-F_{15}L_{15}F_{25}R_1$, $F_{16}L_{16}F_{25}R_1$, or $-F_{18}L_{18}F_{25}R_1$, respectively, wherein F_{25} is $-NH-$, $-NH(C{=}S)NH$, or $-NH(C{=}O)NH$. Examples of compounds which include moiety R_1 and which are suitable for such

nucleophilic reaction are the known compounds, EDTA anhydride and DTPA anhydride (Chu and Orgel, 1985, supra); and 1-(p-isothiocyanato-phenyl)EDTA (herein PITCP-EDTA) (Hemmila et al., 1984, supra); 1-(p-isothiocyanato-benzyl)EDTA (herein PITCB-EDTA) (Meares et al., Anal. Biochem. 142, 68-78 (1984); 1-(p-isocyanato-phenyl)EDTA (herein PICP-EDTA) and 1-(p-isocyanato-benzyl)EDTA herein PICB-EDTA) can also be employed. EDTA and DTPA is also suitable for the reaction, provided that a water soluble carbodiimide coupling reagent, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide, is present in the reaction solution. Reaction with EDTA anhydride or DTPA anhydride is in aqueous buffer at a pH between 6 and 8 with the anhydride present at about 10 mg/ml and a 10-fold to 10,000-fold molar excess relative to polynucleotide. Reaction with PITCP-EDTA, PITCB-EDTA, PICP-EDTA, or PICB-EDTA is in aqueous buffer at pH between 8 and 10 with the EDTA derivative in a 10-fold to 1,000-fold molar excess relative to nucleotide. Reaction with EDTA or DTPA is in aqueous buffer at pH 6 to 7 with EDTA or DTPA at a 10-fold to 10,000-fold molar excess relative to nucleotide and carbodiimide at .01 M to .2 M and in large (10-1,000) molar excess relative to EDTA or DTPA.

The probe is isolated from the reaction mixture employing standard chromatographic procedures, particularly HPLC (high performance liquid chromatography) or gel permeation chromatography.

PITCP-EDTA, PITCB-EDTA, PICP-EDTA, PICB-EDTA or DTPA can be complexed with Eu^{+3}, Tb^{+3} or Sm^{+3} and used, in chelate form, in the nucleophilic reaction in essentially the same way as the unchelated form to make probe. Then, in the resulting probe, R_1 will be complexed with the lanthanide III ion.

Alternatively, if EDTA anhydride or DTPA anhydride, or PITCP-EDTA, PITCB-EDTA, PICP-EDTA,

PICB-EDTA, EDTA or DTPA not complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, is employed with the nucleophilic reaction to make probe, R_1 in the resulting probe will not be complexed with lanthanide ion. From this lanthanide ion-free probe, probe that is complexed with the Eu^{+3}, Tb^{+3} or Sm^{+3} is prepared by the following procedure (referred to hereinafter as the "standard probe chelation process"): The lanthanide ion-free probe at between about 1 mg/ml and about 10 mg/ml in a volume of sodium citrate buffer, with citrate concentration between about 0.05 M and about 0.5 M and pH of about 6.5 to about 7, is cooled on ice and is combined with an equal volume of a solution, in HCl at about 0.1 M to about 1 M (about twice the concentration of citrate in the probe solution), of a salt of the lanthanide ion, with a concentration of said salt between about 0.1 times equimolar and between about 25 times equimolar, preferably about 1 time to 2 times equimolar, with respect to the concentration of chelator tag moieties R_1 linked to probe in the solution. Then the pH of the resulting solution is adjusted if necessary to about 3 to about 3.5 by addition of NaOH or HCl and incubated on ice for about 10 to about 20 minutes. Finally, the pH of the solution is increased to neutral (i.e., 6 to 8) by additon of 1 M of NaOH and the solution is briefly (i.e., about one minute) incubated at room temperature. Finally, the labeled probe, complexed with the Eu^{+3}, Tb^{+3} or Sm^{+3}, is isolated from the solution by a standard procedure, e.g., by gel filtration using Sephadex G-50 with 0.1 M to 0.5 M sodium citrate (pH 6.5 to 7). Preferred salts for this purpose are $EuCl_3$, $TbCl_3$ or $SmCl_3$.

As noted above, a polynucleotide with a sequence of probe and comprising a pyrimidine moiety with a group of formula $-CH=CH(CH_2)_vNH_2$, wherein v is 1 to 20, bonded to carbon-5, can be prepared enzymatically by known methods. For example, such a DNA can be prepared by employing E. coli DNA polymerase I and, as template, a double-stranded DNA which comprises, in at least one of

its strands, a target sequence of the target DNA or RNA of the probe, and by carrying out the synthesis with dATP, dCTP, dGTP, TTP, and dUTP or dCTP, wherein the uracil or cytosine moiety was modified to have a group of formula $-CH=CH(CH_2)_vNH_2$ bonded to carbon-5. These analogs of dUTP and dCTP are known compounds or are readily prepared by the skilled employing known techniques. See, e.g., Ward et al., supra. DNA precursors of probes of the invention, which have the sequence of a probe and which comprise a pyrimidine moiety with a group $-CH=CH(CH_2)_vNH_2$ bonded to carbon-5, can also be prepared by known nick-translation methods using the same template, the same polymerase enzyme, and the same deoxyribonucleoside triphosphates including the dUTP or dCTP modified with the $-CH=CH(CH_2)_vNH_2$, but also employing in the reaction mixture a DNAase I, as from bovine pancreas. See Langer et al., supra, and Ward et al., supra.

An RNA precursor of a probe of the invention, wherein one or more pyrimidine moieties are modified to have a group of formula $-CH=CH(CH_2)_vNH_2$ bonded to carbon-5, can also be prepared enzymatically by known methods, employing a double-stranded DNA template, wherein at least one of the strands comprises a target sequence of the target DNA or RNA of the probe, a DNA-dependent RNA polymerase such as from E. coli or bacteriophage T7, the ribonucleoside triphosphates ATP, CTP , GTP, and UTP, and UTP or CTP modified to have the group $-CH=CH(CH_2)_vNH_2$ bonded to carbon-5 of the uracil or cytosine moiety. See Langer et al., supra. and Ward et al., supra. The UTP and CTP analogs, like their dUTP and dCTP counterparts, are known compounds or are readily prepared by the skilled.

The preferred moiety of formula $-CH=CH(CH_2)_vNH_2$ bound to carbon-5 of uracil or cytosine in dUTP's, dCTP's, UTP's or CTP's employed in the above-described enzymatic methods to make precursors of probes of the invention is the moiety wherein v is 1.

The enzymatic methods can be employed to make probe of the invention directly by employing, in place of the pyrimidine deoxyribonucleoside triphosphate or pyrimidine ribonucleoside triphosphate modified to have $-CH=CH(CH_2)_vNH_2$ bonded to carbon-5, dUTP, dCTP, UTP or CTP modified to have bonded to carbon-5 a group of formula $-CH=CH(CH_2)_vF_{26}R_{26}$, wherein v is 1 to 20, preferably 1, and wherein $-F_{26}R_{26}$ is $-NHR_{261}$, $-NH(C=O)NHR_{262}$- or $-NH(C=S)NHR_{262}$, preferably $-NHR_{261}$, and wherein R_{261} is EDTAyl or DTPAyl and R_{262} is p-EDTA-phenyl or p-EDTA-benzyl, and R_{261} and R_{262} are optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

dUTP or dCTP, wherein the uracil or cytosine has bonded to carbon-5 a group of formula $-CH=CH(CH_2)_vF_{26}R_{26}$, defined as in the preceding paragraph, is a substrate for extension of DNA strands, from 3'-terminal-2'-deoxynucleotides wherein the 3'-carbon is hydroxylated, with the enzyme terminal deoxynucleotidyl transferase ("TdT"). This enzyme, well known in the genetic engineering art, can be obtained, for example, from bovine calf thymus. Brakel et al., supra, describe the use of TdT to extend DNAs, from 3'-terminal nucleotides with hydroxylated 3'-carbon atoms, with dUTP's wherein the uracil has bonded to carbon-5 a group of formula $-CH=CH(CH_2)_vNH(\text{biotinyl})$, wherein v is 1 to about 20. The methods of Brackel et al., supra, are found to be operable also with the modified dUTP's and dCTP's described above in this paragraph in place of the modified dUTP's employed by Brakel et al., supra. Thus, a probe of the invention can be prepared by providing a double-stranded DNA, wherein at least one of the strands comprises a target sequence of a target DNA or RNA of a probe, or a single-stranded DNA, which comprises a target sequence of a target DNA or RNA of a probe, said double-stranded or single-stranded DNA having a 3'-hydroxyl at the 3'-terminal nucleotide of

said strand which comprises a target sequence of target DNA or RNA, and extending said 3'-hydroxy terminated strand, in a TdT-catalyzed reaction, with dUTP or dCTP wherein the uracil has bonded to carbon-5 a group of formula $-CH=CH(CH_2)_vF_{26}R_{26}$, and, optionally, other, unmodified 2'-deoxynucleoside triphosphates.

In the above-described methods for making probe of the invention by TdT-catalyzed DNA strand extension, the group R_{26} on the modified dUTP is optionally, and preferably, complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}; the preferred groups bonded to carbon-5 of the modified dUTP or dCTP employed in the extension reaction are $-CH=CH(CH_2)NH(EDTAyl)$ and $-CH=CH(CH_2)NH(DTPAyl)$; and the extension is carried out preferably so that, on the average, between 1 and 5 modified uridines or cytidines are added to the 3'-terminus of each substrate strand. The preferred TdT is from calf thymus.

In all of the above-described enzymatic methods for making probe, metal ions such as Mg^{+2}, Mn^{+2} or Co^{+2} must be present for enzymatic activity, as known in the art. For example, if a double-stranded DNA employed as template for chain extension with TdT has a strand with a recessed 3'-terminus, Co^{+2} must be present or the TdT will not catalyze extension of said strand. These metal ions, e.g., Mg^{+2}, Mn^{+2}, Co^{+2}, are chelated by tag moiety-chelators of formula $-R_{261}$ or R_{262}. Consequenty if UTP, CTP, dUTP or dCTP modified to have a group of formula $-CH=CH(CH_2)_vF_{26}R_{26}$ wherein R_{26} is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, is employed in an above-described enzymatic method to make probe, the Eu^{+3}, Tb^{+3} and Sm^{+3} of at least a fraction of the groups R_{26} will be replaced with metal ion required for enzymatic activity. Further, if the group R_{26} linked to the modified UTP, CTP, dUTP or dCTP employed in the enzymatic reaction is not complexed with metal ion, it will chelate metal ion that must be present in the enzyme reaction mixture for enzymatic activity. Thus, if probe to be made by one of the above-described

enzymatic reactions is intended to have tag moiety not complexed with metal ion, and UTP, CTP, dCTP or dUTP wherein the uracil or cytosine has bonded to carbon-5 a group of formula $-CH=CH(CH_2)_vF_{26}R_{26}$ is employed in the enzymatic reaction, the probe isolated from the reaction mixture must be treated to separate metal ion from the tag moieties. This can be accomplished, for example, by dialyzing solution with the probe against metal-free buffer using standard procedures known in the art. If probe is to be made by one of the above-described enzymatic methods and is intended to have tag moiety complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, and if UTP, CTP, dUTP or dCTP, wherein the uracil or cytosine has bonded to carbon-5 a group of formula $-CH=CH(CH_2)_vF_{26}R_{26}$, is employed in the enzymatic reaction, the probe as isolated from the enzyme reaction mixture, whether or not R_{26} on the UTP, CTP, dUTP or dCTP used in the enzyme reaction was complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, will be treated by the standard probe chelation process described above.

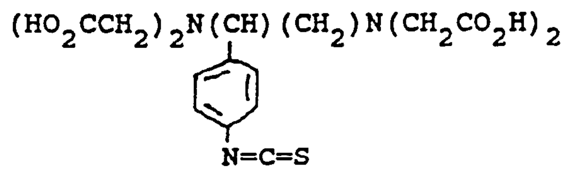
To prepare dUTP, dCTP, UTP or CTP wherein a group of formula $-CH=CH(CH_2)_vF_{26}R_{26}$ is bonded to carbon-5 of the uracil moiety, the following methods are used, starting with the known dUTP, dCTP, UTP or CTP modified to have the group of formula $-CH=CH(CH_2)_vNH_2$ bonded to carbon-5 of the uracil or cytosine moiety (See Langer et al. (1981), supra, and Ward et al., supra).

If R_{26} is R_{261} (i.e., EDTAyl or DTPAyl), the dUTP, dCTP, UTP or CTP with uracil or cytosine with $-CH=CH(CH_2)_vNH_2$ bonded to the 5-position is reacted at room temperature in aqueous solution, buffered to a pH of about 6 to 8, with the known EDTA anhydride or DTPA anhydride (see Chu and Orgel, Proc. Natl. Acad. Sci. 82, 963 (1985)).

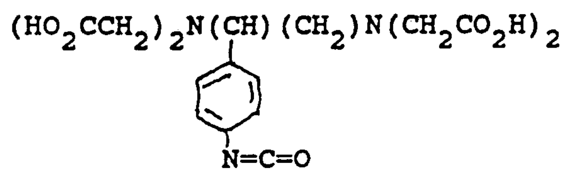
Alternatively, if R_{26} is R_{261}, EDTA or DTPA can be reacted directly with the $-CH=CH(CH_2)_vNH_2$-derivatized ribonucleotide or

2'-deoxyribonucleotide in the presence of a water-soluble carbodiimide coupling reagent, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, at about pH 6 to 7, with the carbodiimide at about 0.01 M to 0.2 M and large molar excess relative to both nucleotide and EDTA or DTPA.

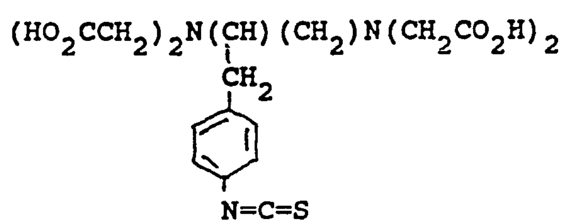
If $-R_{26}$ is p-EDTA-phenyl or p-EDTA-benzyl , the dUTP, dCTP, UTP or CTP with uracil or cytosine with $-CH=CH(CH_2)_vNH_2$ bonded to position-5 is reacted in aqueous solution buffered to a pH between about 8 and about 10 with the known 1-(p-isothiocyanato-phenyl) EDTA (PITCP-EDTA) of formula



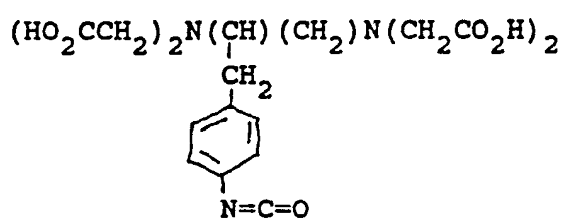
(see Hemmila, Anal. Biochem. 137, 335-343 (1984)) or the 1-(p-isocyanato-phenyl) EDTA (PICP-EDTA) of formula



the known 1-(p-isothiocyanato-benzyl)EDTA (PITCB-EDTA) of formula



(see Meares et al., Anal. Biochem. 142, 68-78 (1984), or 1-(p-isocyanato-benzyl) EDTA (PICB-EDTA) of formula



The PICP-EDTA is prepared following the procedure of Hemmila et al. (1984), supra, for preparation of PITCP-EDTA by condensing PDP-EDTA (see Example IV, below) in a water-chloroform mixture with phosgene, removing the aqueous layer, and isolating the PICP-EDTA from the aqueous layer by drying.

The PICB-EDTA is prepared following the method of Meares et al., Anal. Biochem. 142, 68-78 (1984), for preparing PITCB-EDTA. p-Aminobenzyl EDTA is condensed in a water chloroform mixture with phosgene, the aqueous layer is removed and the PICB-EDTA is isolated from the aqueous layer by drying.

dUTP, dCTP, UTP or CTP wherein the group R_{26} linked to uracil or cytosine through the carbon-5 is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, even though not polynucleotides, can be prepared by first making dUTP, dCTP, UTP or CTP wherein the group $-CH{=}CH(CH_2)_vF_{26}R_{26}$ is bonded to carbon-5 and then subjecting said dUTP, dCTP, UTP or CTP to the standard probe chelation process.

Alternatively, the chelate of DTPA, PITCP-EDTA, PICP-EDTA, PITCB-EDTA or PICB-EDTA with Eu^{+3}, Tb^{+3} or Sm^{+3} can be prepared and said chelate employed, in place of the corresponding compound without lanthanide ion bound, in the reaction with dUTP, dCTP, UTP or CTP, wherein the uracil or cytosine is derivatized at carbon-5 with $-CH{=}CH(CH_2)_vNH_2$, to prepare directly dUTP, dCTP, UTP or CTP wherein the tag moiety is complexed with the lanthanide ion. DTPA chelates of Eu^{+3}, Tb^{+3} and Sm^{+3} are known. PITCP-EDTA complexed with Eu^{+3} is known (see Hemmila et al. (1984), supra). This compound complexed with Tb^{+3} or Sm^{+3} is made in the same way as the Eu^{+3} complex except that $TbCl_3$ or $SmCl_3$ is employed in place of $EuCl_3$. The lanthanide ion complexes of PICP-EDTA, PITCB-EDTA and PICB-EDTA are prepared in the same way as the lanthanide ion complexes of PITCP-EDTA.

Any of the methods described below for preparing a double-stranded DNA which comprises a DNA with sequence

of a probe can be applied to provide a double-stranded DNA template for use in the above-described methods for preparing, by DNA polymerase-, RNA polymerase- or TdT-catalyzed nucleic acid synthesis, a probe of the invention comprising a modified uracil or cytosine moiety. Similarly, the methods described below for preparing a single-stranded DNA with sequence of a probe can be used to supply a single-stranded DNA substrate for preparation with TdT of a probe of the invention comprising a modified uracil or cytosine moiety.

Thus, one method of the invention for making a probe of the invention comprises providing a precursor polynucleotide, which is a polynucleotide which has the sequence of the probe and which comprises a nucleoside base bonded to a linker moiety of formula $-F_1L_1NH_2$ and (i) reacting said polynucleotide with a compound selected from EDTA anhydride, DTPA anhydride, PITCP-EDTA, PITCB-EDTA, PICB-EDTA and PICP-EDTA, wherein the PITCP-EDTA, PITCB-EDTA, PICB-EDTA or PICB-EDTA is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3} or (ii) in aqueous solution buffered to a pH of 6 to 7, reacting said polynucleotide with EDTA or DTPA, wherein the DTPA is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, with a water soluble carbodiimide coupling agent. The preferred coupling agent for this purpose is 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. However, any water soluble carbodiimide coupling agent known in the art can be employed, such as, for example, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide. Numerous methods of providing the polynucleotide are available, as described above. If the probe obtained by one of the above reactions is not complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, a probe that is so complexed is obtained, usually after purification by HPLC or gel permeation chromatography, by carrying out the above-described standard probe chelation process with the uncomplexed probe. If DTPA, PICP-EDTA, PITCP-EDTA PITCB-EDTA or PICB-EDTA complexed with lanthanide ion is employed in

the reaction, the eluant employed in chromatographic isolation of the resulting probe will include preferably sodium citrate at about 0.1 M-0.5 M and pH 6.5 to 7 or, alternatively, DTPA (or EDTA) at about 10 um-100 uM with $CaCl_2$ at about twice the DTPA or EDTA concentration, in order to remove from purified probe any lanthanide ion freed during the reaction and not complexed with EDTA or DTPA tag moiety on the probe.

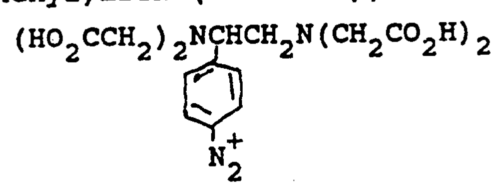
In this process of the invention, the group $-F_1L_1NH_2$ is preferably bonded to the 8-position of a purine moiety, wherein it is preferably of formula $-NH(CH_2)_tNH_2$ wherein t is 2 to 8, or to the 5-position of a pyrimidine moiety, wherein it is preferably of formula $-CH{=}CHCH_2NH_2$. Most preferably, the moiety is uracil.

Another method of the invention for making a probe of the invention, which probe comprises a uracil or cytosine moiety bonded through carbon-5 to a group of formula $-CH{=}CH(CH_2)_vF_{26}R_{26}$, wherein v is 1 to 20; $F_{26}R_{26}$ is $-NHR_{261}$, $-NH(C{=}S)NHR_{262}$ or $-NH(C{=}O)NHR_{262}$; R_{261} is EDTAyl or DTPAyl; and R_{262} is p-EDTA-phenyl or p-EDTA-benzyl, and R_{26} is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, comprises (A) providing (i) a linear double-stranded DNA, at least one strand of which has a hydroxyl group bonded to the 3'-terminal carbon or (ii) a linear single-stranded DNA with a hydroxyl group bonded to the 3'-terminal carbon; (B) extending the strand or strands of said linear double-stranded DNA which have a 3'-terminal nucleotide with a 3'-hydroxyl group or said linear single-stranded DNA in a TdT-catalyzed reaction to make a polynucleotide with the sequence of the probe, employing as a substrate in said strand-extension a dUTP or dCTP wherein the uracil or cytosine moiety is bonded through carbon-5 to a group of formula $-CH{=}CH(CH_2)_vF_{26}R_{26}$, wherein

R_{26} is optionally complexed with Eu^{+3}, Tb^{+3} or

Sm^{+3}; and (C)(i) if R_{26} in probe is not complexed with metal ion, dialyzing the product of said reaction against a metal-free buffer or (ii) if R_{26} in probe is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, carrying out with the product of said reaction the standard probe chelation process. The most preferred substrates for this method of making probe by TdT-catalyzed chain-extension are dUTP with a group of formula $-CH{=}CHCH_2NH(R_{261})$ bonded to carbon-5 of uracil, wherein R_{261} is complexed with Eu^{+3}. The product is preferably isolated employing step (C)(ii). A DNA segment extended in the reaction preferably comprises, prior to the reaction, a probing sequence suitable for the target DNA or RNA of the probe, although such a probing sequence can be made in the extension reaction. Preferably only modified dUTP or dCTP (or both) will be employed as substrate in the extension reaction, and the reaction will be carried out so that, on the average, 1 to 5 modified nucleotides are added to the 3′-terminus of each extended polynucleotide. The method can be employed advantageously with single-stranded DNA, from an automated synthesizer, that is about 12 to about 100 nucleotides long.

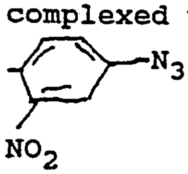
We have also discovered another method of the invention to prepare probe of the invention. We have found that probe can be made by simply reacting 1-(p-diazo-phenyl)EDTA (PDP-EDTA), of formula:



optionally complexed with Eu^{+3}, Tb^{+3} and Sm^{+3}, with a polynucleotide with the sequence of a probe. This process yields a nucleic acid probe according to the invention wherein the DNA or RNA is non-specifically labeled with p-EDTA-phenyl, complexed with Eu^{+3}, Tb^{+3}

or Sm^{+3} if the PDP-EDTA was, as preferred, so complexed, as a result of the nucleophilic displacement by nucleophiles on the polynucleotide of N_2 from the diazo phenyl of the PDP-EDTA under neutral to alkaline conditions. PDP-EDTA and its chelates with Eu^{+3} and Tb^{+3} are known, Sundberg et al., J. Med. Chem. 17, pp. 1304-1307 (1974); Leung and Meares, Biochem. Biophys. Res. Commun. 75, pp. 149-155 (1977); Hemmila et al., supra. See also Example IV below. The PDP-EDTA chelate of Sm^{+3} is prepared in the same way as that of Eu^{+3} or Tb^{+3} but employing $SmCl_3$ in place of $EuCl_3$ or $TbCl_3$.

In yet another method of the invention for making probe of the invention, a phenyl-azide-derivatized EDTA or DTPA of formula $(R_{263})NH(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}NH(R_{261})$, wherein R_{261} is EDTAyl or DTPAyl and is optionally (and preferably) complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, R_{263} is



, R_{264} is hydrogen or n-alkyl of 1 to 3 carbon atoms, aa is 1 to 6, bb is 1 to 6 and cc is 0 or 1 is reacted under photoactivating conditions with a polynucleotide with a sequence of a probe. This process yields a nucleic acid probe according to the invention wherein the DNA or RNA is non-specifically labeled as a result of reaction with the nitrene which results from photolysis of the azide. If the phenyl azide derivative employed in the reaction was complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, the probe resulting from the reaction will be so complexed as well. "Photoactivating conditions" simply require that the solution of polynucleotide with sequence of the probe and of phenyl-azide-derivatized EDTA or DTPA (optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}) be illuminated with light of wavelength low enough to photolyze the phenyl azide to a phenyl nitrene and preferably high

enough to avoid damage to the polynucleotide from ultraviolet light. Wavelengths between about 340 nm and 380 nm are suitable.

The preparation of phenyl-azide-derivatized EDTA's and DTPA's of the invention is illustrated in Example XI with the compound wherein R_{261} is DTPAyl, R_{264} is $-CH_3$, aa is 3, bb is 3, and cc is 1. The preparation, carried out in the dark, follows that of Forster et al., supra, for phenyl-azide-derivatized biotin except that DTPA anhydride or EDTA anhydride is employed in place of N-hydroxysuccinimide ester of biotin in the reaction with the amino-derivatized 4-fluoro-3-nitrophenyl azide. The phenyl azide-derivatized DTPA or EDTA can be complexed with Eu^{+3}, Tb^{+3} or Sm^{+3} by the same method as PDP-EDTA, but carried out in the dark.

In the non-specific labeling processes of the invention, single-stranded polynucleotide is preferably employed. The process is illustrated in Example V for PDP-EDTA and Example XII for phenyl azide-derivatized EDTA or DTPA. The process is carried out with an initial molar concentration of PDP-EDTA, or phenyl-azide-derivatized EDTA or DTPA, of between about 0.1 X and 2 X the molar concentration of deoxyribonucleotides or ribonucleotides in the polynucleotide with sequence of probe that is to be labeled in the reaction. Any of the processes described below for providing a polynucleotide with sequence of probe can be employed to provide polynucleotide to be labeled by the process of reacting with PDP-EDTA, optionally and preferably complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, or with phenyl-azide-derivatized EDTA or DTPA of formula $(R_{263})NH(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}NH(R_{261})$, wherein R_{261}, R_{263}, R_{264}, aa, bb and cc are as defined above and the compound is optionally and preferably complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}. The reaction is carried out by combining an aqueous solution of polynucleotide, preferably single-stranded, at between about 0.001 mg/ml

and 3 mg/ml concentration, with an aqueous solution of the PDP-EDTA or phenyl-azide-derivatized EDTA or DTPA, at between about 0.3 uM and 2 mM (about 0.1 X to 2 X the molar concentration of nucleotides) and allowing the reaction to proceed at 0°C to 10°C for between about 1 hour and 8 hours at a pH between about 7.5 and 8.5 (with PDP-EDTA) or about 6 and 8 (with the phenyl azide-derivatized EDA or DTPA). The reaction with phenyl-azide-derivatized EDTA or DTPA occurs under photoactivating conditions. After the reaction, the probe, if the reaction was run with PDP-EDTA or phenyl azide-derivatized EDTA or DTPA, not complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, is purified from the reaction mixture (a) chromatographically, preferably by gel permeation chromatography on, for example, Sephadex G-50, using a buffer such as 0.01 M Tris-HCl at a pH between about 7 and about 8 as eluant or (b) by precipitation, as with ethanol. If the reaction between polynucleotide and PDP-EDTA or phenyl azide-derivatized DTPA or EDTA was carried out with the PDP-EDTA or phenyl-azide-derivatized DTPA or EDTA complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, the chromatographic purification of probe will be by gel permeation chromatography employing, for example, Sephadex G-50 and 0.1 M to 0.5 M sodium citrate, pH 6.5 to 7, as eluant. The citrate eluant serves to complex any dissociated lanthanide ion and separate it from probe being purified. An alternative, but less preferred, eluant to accomplish this purpose of separating dissociated lanthanide ion from probe, is about 10 uM to about 100 uM DTPA or EDTA with an approximately 2-fold molar excess, relative to DTPA or EDTA, of a calcium salt, such as $CaCl_2$.

If the desired probe of the invention is complexed with a lanthanide III ion, but the PDP-EDTA, or phenyl azide-derivatized EDTA or DTPA, used to non-specifically label polynucleotide with sequence of probe is not so complexed, the probe obtained from the reaction between PDP-EDTA, or phenyl-azide-derivatized

EDTA or DTPA, and polynucleotide is, after purification by chromatography or precipitation as described above, subjected to the standard probe chelation process with a salt of Eu^{+3}, Tb^{+3} or Sm^{+3}.

The reaction between PDP-EDTA, or phenyl azide-derivatized EDTA or DTPA, and polynucleotide is carried out so that between about 1 in 12 and about 1 in 1,000, most preferably about 1 in 100, nucleotides in the probe is labeled. The extent of labeling under given reaction conditions can be determined by spectroscopic and other analytical techniques well known in the art and reaction conditions can be adjusted appropriately to achieve a desired extent of labeling. With both the PDP-EDTA and phenyl azide-derivatized compounds, the extent of labeling can be determined by forming a lanthanide III ion (e.g., Eu^{+3}) complex with the non-specifically labeled polynucleotide and then measuring the amount of chelated lanthanide III ion by extracting, from a known quantity of the labeled polynucleotide, the ion employing a fluorescence enhancement solution, described below, and comparing the fluorescence intensity from the resulting solution with that from comparable standard solutions which have known concentrations of the lanthanide ion. In the case of the phenyl azide-derivatized compounds, between about 1% and 3% of the phenyl azide derivative in solution reacts with polynucleotide. See, e.g., Staros, Trends in Biochemical Sciences 5, 320-322 (1980); and Forster et al., supra. This fact can be used to estimate concentrations necessary to achieve desired extent of labeling.

The methods of the invention for preparing probe by non-specific reaction with PDP-EDTA (optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}), or phenyl azide-derivatized EDTA or DTPA (also optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}), is preferably carried out with probes between about 100 and 10,000 nucleotides in length.

Other methods of the invention for making nucleic acid probe of the invention, described below, use as starting material a nucleic acid (DNA or RNA) with sequence of probe which has:

(i) a 5'-terminal carbon bonded to a group of formula

$$-\mathrm{O}-\overset{\mathrm{O}}{\overset{\|}{\underset{\mathrm{O}}{\underset{|}{\mathrm{P}}}}}-\mathrm{NH}-\mathrm{L}_5-\mathrm{NH}_2; \quad -\mathrm{O}-\overset{\mathrm{O}}{\overset{\|}{\underset{\mathrm{O}}{\underset{|}{\mathrm{P}}}}}-\mathrm{NH}-\mathrm{L}_5-\mathrm{SH} \quad -\mathrm{O}-\overset{\mathrm{O}}{\overset{\|}{\underset{\mathrm{O}}{\underset{|}{\mathrm{P}}}}}-\mathrm{O}-\mathrm{L}_6-\mathrm{SS}-\mathrm{L}_5-\mathrm{NH}_2;$$

$$-\mathrm{O}-\overset{\mathrm{O}}{\overset{\|}{\underset{\mathrm{O}}{\underset{|}{\mathrm{P}}}}}-\mathrm{S}-\mathrm{CH}_2(\mathrm{CO})\mathrm{L}_5\mathrm{NH}_2; \quad \text{or } -\mathrm{NH}_2,$$

wherein L_5 is alkyl of 2 to 20 carbon atoms, and L_6 is alkyl of 3 to 20 carbon atoms; or (ii) a 3'-terminal carbon bonded to a group of formula

$$-\mathrm{O}-\overset{\mathrm{O}}{\overset{\|}{\underset{\mathrm{O}}{\underset{|}{\mathrm{P}}}}}-\mathrm{NH}-\mathrm{L}_5-\mathrm{NH}_2 \qquad -\mathrm{O}-\overset{\mathrm{O}}{\overset{\|}{\underset{\mathrm{O}}{\underset{|}{\mathrm{P}}}}}-\mathrm{NH}-\mathrm{L}_5-\mathrm{SH},$$

or $-\mathrm{O}-\overset{\mathrm{O}}{\overset{\|}{\underset{\mathrm{O}}{\underset{|}{\mathrm{P}}}}}-\mathrm{S}-\mathrm{CH}_2(\mathrm{CO})\mathrm{L}_5\mathrm{NH}_2$, wherein $-L_5$ is alkyl of 2 to 20 carbon atoms.

These methods, employing nucleic acids with modified terminal nucleotides, are preferably employed to make probes, between about 10 and about 100 nucleotides in length, which are based on nucleic acids that can be synthesized advantageously by automated, stepwise solid phase methodology. The more preferred of the methods employ nucleic acids with modified 5'-terminal nucleotides.

Nucleic acids with the modified terminal nucleotides described above, and employed as starting materials in methods of the invention to prepare probes of the invention, are known.

A nucleic acid with a 5'-terminal nucleotide modified to have a group of formula $-OPO_2(NH)L_5NH_2$

bonded to the 5'-carbon can be prepared by the methods of Chu et al., Nucleic Acids Research 11, 6513-6529 (1983); see also Chu and Orgel, Proc. Natl. Acad. Sci. 82, 963-967 (1985). The methods of Chu et al. (1983), supra, and Chu and Orgel (1985), supra, can also be employed to prepare a nucleic acid with a group of formula $-OPO_2(NH)L_5NH_2$ bonded to the 3'-carbon of the 3'-terminal nucleotide. First, the single-stranded nucleic acid with the desired sequence and with a phosphate group bonded to the 3'-terminal carbon or the 5'-terminal carbon is provided. This nucleic acid is then reacted for 2-4 hours at room temperature in the presence of approximately 0.1 M imidazole-HCl buffer (about pH 6) and approximately 0.1 M of a water soluble carbodiimide coupling agent, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, to form the phosphoroimidazolide derivative. The phosphoroimidazolide derivative is isolated by HPLC and is then reacted for 2-4 hours at 50°C and at a pH between about 7 and about 8 with a diamine of formula $H_2NL_5NH_2$, at a concentration of between about 0.05 M and 0.5 M, to form the desired derivative with $-OPO_2(NH)L_5NH_2$ bonded to the 3'-terminal carbon or the 5'-terminal carbon. This derivative is purified by HPLC. In an alternative procedure, the nucleic acid with the 3'-terminal carbon or 5'-terminal carbon bonded to a phosphate group is combined with 0.05 M to 0.5 M diamine of formula $H_2NL_5NH_2$, approximately 0.1 M methylimidazole.HCl buffer (about pH 6) and approximatel 0.1 M of a water soluble carbodiimide coupling agent, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, the mixture is incubated for 12-20 hours at room temperature, and the desired polynucleotide, derivatized at the carbon with a group of formula $-OPO_2(NH)L_5NH_2$, is purified by HPLC. L_5, in nucleic acids derivatized with $-OPO_2(NH)L_5NH_2$, is preferably n-alkyl of 2 to 8 carbons.

A nucleic acid with a group of formula $-OPO_2(NH)L_5SH$ bonded to the 5′-terminal carbon is prepared by a method adapted from that of Chu et al. Nucl. Acids Res. 14,5591-5603 (1986). This method can also be employed to prepare a nucleic acid with a group of formula $-OPO_2(NH)L_5SH$ bonded to the 3′-carbon of the 3′-terminal nucleotide. First, the single-stranded nucleic acid with the desired sequence and with a phosphate group bonded to the 5′-terminal carbon or the 3′-terminal carbon is provided. The phosphoroimidazolide derivative of the nucleic acid is formed and is isolated by HPLC as described above in connection with preparing the $-OPO_2(NH)L_5NH_2$ derivatized nucleic acid. The phosphoroimidazolide derivative (between about 10 ug and 30 ug) is collected in 300 ul of 100 mM NaCl, 1mM EDTA and 10 mM HEPES, pH 7.3. To this solution is added aqueous dihydrochloride of compound of formula $NH_2L_5SSL_5NH_2$ (e.g., cystamine dihydrochloride), at about 1M, to a final concentration of 250 mM. The resulting solution is incubated for 1 to 3 hours at 50°C. The derivatized nucleic acid is then isolated by ethanol precipitation. Between about 100 ng and about 50 ug of the derivatized nucleic acid is then dissolved in 100-200 ul of 0.1M dithiothreitol (DTT), 1mM EDTA, 10mM HEPES, pH 7.7, and the solution is incubated at 23°C for 1 hour. The resulting nucleic acid, derivatized with $-OPO_2(NH)L_5SH$, is isolated by HPLC and is stored in 0.01M DTT, 10mM HEPES, pH 7.7, to prevent dimerization through disulfide formation, until further derivatization with p-EDTA-phenyl or p-EDTA-benzyl as described below. In the nucleic acids derivatized with $-OPO_2(NH)L_5SH$, it is preferred that L_5 be n-alkyl of 2 to 6 carbon atoms.

These methods, of derivatizing with $-OPO_2(NH)L_5NH_2$ or $-OPO_2(NH)L_5SH$, can be employed on mixtures of polynucleotides, some of which have a 5′-phosphate on the 5′-terminal carbon, and some

of which have a 3'-phosphate on the 3'-terminal carbon, to yield a mixture of $-OPO_2(NH)L_5NH_2$-terminated or $-OPO_2(NH)L_5SH$-terminated polynucleotides. The methods can also be used on polynucleotides wherein both terminal nucleotides are phosphorylated, at the 5'-terminal carbon and the 3'-terminal carbon, to yield polynucleotides terminated at both ends with $-OPO_2(NH)L_5NH_2$ or $-OPO_2(NH)L_5SH$. For example, the methods could be applied to a mixture of polynucleotides, some with 5'-terminal-5'-phosphates, some with 3'-terminal-3'-phosphates, and some with both 5'-terminal-5'-phosphates and 3'-terminal-3'-phosphates, resulting from random cleavage of polynucleotide, as by sonication.

The preferred phosphate-terminated polynucleotides for use in the invention are those with phosphate bonded to the 5'-terminal carbon. These are conveniently prepared by first preparing a polynucleotide with the desired sequence of probe by an automated, stepwise, solid phase synthesis procedure and then 5'-phosphorylating the polynucleotide using standard procedures with T4 polynucleotide kinase. Polynucleotides phosphorylated with T4 polynucleotide kinase will have 3'-terminal nucleotides with hydroxylated 3'-carbons and thus can be employed to make probe with TdT, with T4 RNA ligase, or with TdT followed by T4 RNA ligase as described elsewhere herein.

A nucleic acid with a group of formula $-OPO_2SCH_2(CO)L_5NH_2$ bonded to the 5'-terminal carbon is prepared in two steps.

First, employing T4 polynucleotide kinase with the nucleic acid with an hydroxyl group on the 5'-terminal carbon and with the known, 5'-gamma-thiophosphate analog of ATP (i.e., wherein the gamma phosphate is replaced with $-OPSO_2H$) in place of ATP, the thiophosphate group is bonded to the 5'-terminal carbon. (The group of

formula $-O\overset{\overset{S}{\|}}{P}O_2H$ is referred to herein as "thiophosphate".) Conditions for this T4 polynucleotide kinase-catalyzed reaction are the same as the known conditions that would be employed if ATP were the substrate.

Second, the nucleic acid so modified is reacted with an alpha-haloketone derivative of formula $H_2NL_5(CO)CH_2X_5$, wherein X_5 is chloro or bromo, under conditions known to, or readily ascertained by, the skilled to be suitable for nucleophilic displacement of the halogen by the sulfur of the thiophosphate. The compounds of formula $H_2NL_5(CO)CH_2X_5$ are known or readily synthesized by the skilled using known methods.

A nucleic acid with a group of formula $-OPO_2SCH_2(CO)L_5NH_2$ bonded to the 3′-terminal carbon is prepared in either of two ways, based on modifications of the teaching of Cosstick et al., Nucl. Acids Rsch. 12, 1791-1800 (1984). Both of the methods employ the known enzyme T4 RNA ligase and, as nucleic acid substrate, a polynucleotide with a ribonucleotide at its 3′-terminus, said ribonucleoside having a hydroxyl group bonded to its 3′-carbon. Such a polynucleotide can be either RNA or DNA with such a ribonucleoside at its 3′-terminus. As known in the art, a DNA with a hydroxyl bonded to its 3′-terminal carbon can be ligated, through said hydroxyl, to a ribonucleoside-5′-phosphate in a reaction catalyzed by TdT. In the first of the methods, following Cosstick et al., supra, a 2′-deoxyribonucleoside-5′-phosphate-3′-thiophosphate (synthesized as taught in Cosstick et al.) is ligated to the 3′-terminus of the polynucleotide with the 3′-terminal ribonucleoside in a reaction catalyzed by T4 RNA ligase. Then, the resulting polynucleotide, with the group of formula $-O\overset{\overset{S}{\|}}{P}O_2H$ bonded to the 3′-carbon of the 3′-terminal 2′-deoxyribonucleotide, is reacted with a compound of

formula $H_2NL_5(CO)CH_2X_5$ in the same way as described above for polynucleotides with thiophosphate at the 5'-terminal carbon. In the second of the methods, which is part of our invention and can be employed to make polynucleotide with $-OPO_2S(CH_2)(CO)L_5NH_2$ at the 3'-terminal carbon, but is more general and can be employed to make probe directly, the novel 2'-deoxyribonucleotide-5'-phosphate with the group of formula $-OPO_2SCH_2(CO)L_5F_{28}R_{28}$, wherein $-F_{28}R_{28}$ is $-NH_2$, $-NHR_{281}$, $-NH(C{=}S)NHR_{282}$ or $-NH(C{=}O)NHR_{282}$, wherein R_{281} is EDTAyl or DTPAyl, $-R_{282}$ is p-EDTA-phenyl or p-EDTA-benzyl, and R_{281} and R_{282} are optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, bonded to the 3'-carbon is used as a substrate for the T4 RNA ligase. When $-F_{28}R_{28}$ is $-NH_2$, the novel compound is prepared by reacting the corresponding 2'-deoxyribonucleotide-5'-phosphate-3'-thiophosphate with the compound of formula $H_2NL_5(CO)CH_2X_5$ as follows:

The 2'-deoxyribonucleoside-5'-phosphate-3'-thiophosphate is dissolved to give a 1 uM to 10 uM solution in .05 M aqueous HEPES, pH 7. To 1 ml of the solution is added with stirring 10-20 ul of an acetonitrile solution that is 1 mM in compound of formula $H_2NL_5(CO)CH_2X_5$. Stirring is continued at room temperature for 1 hour. The solution is then diluted to 4 ml with water and the desired product isolated chromatographically.

When $-F_{28}R_{28}$ of the novel compound is $-NHR_{281}$, the derivative wherein $-F_{28}R_{28}$ is NH_2 is reacted with excess EDTA anhydride or DTPA anhydride in aqueous solution buffered to pH 6 to 8 or, with EDTA or DTPA directly in the presence of excess water-soluble carbodiimide coupling reagent in an aqueous solution buffered to pH 6 to 7. The desired product is isolated chromatographically. If R_{281} is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, the product from reaction with EDTA

anhydride, DTPA anhydride, or EDTA or DTPA not complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, is subjected to the standard probe chelation process; or the product from reaction with EDTA or DTPA complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, is purified using 0.1-0.5 M sodium citrate, pH 6.5-7, as eluant in the chromatography.

When $-R_{28}$ is p-EDTA-phenyl or p-EDTA-benzyl, the derivative wherein $-F_{28}R_{28}$ is $-NH_2$ is reacted with excess PITCP-EDTA, PICP-EDTA, PITCB-EDTA or PICB-EDTA optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, in aqueous buffer at pH 8 to 10. When the p-EDTA-phenyl or p-EDTA-benzyl of the product is not complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, the reactant PITCP-EDTA, PICP-EDTA, PITCB-EDTA or PICB-EDTA is not so complexed and the product is isolated chromatographically. When the p-EDTA-phenyl or p-EDTA-benzyl of the product is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, and the reactant is not, the product of the reaction is subjected to the standard probe chelation process. When the p-EDTA-phenyl or p-EDTA-benzyl of the product and the reactant are complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, the chromatographic purification of product employs 0.1 M-0.5 M sodium citrate, pH 6.5-7 as eluant.

The novel 3'-thiophosphate adducts of the 5'-phosphate -2'-deoxyribonucleoside, wherein the group of formula $-OPO_2SCH_2(CO)L_5F_{28}R_{28}$ is bonded to the 3'-carbon, is another aspect of our present invention, as are the various salts (e.g., with alkali metal ions or Mg^{+2}), acid and base forms, and hydrates of the novel compounds, all of which can be prepared easily by the skilled. The adducts are substrates for the T4 RNA ligase. In a reaction, catalyzed by the ligase, between a polynucleotide with a 3'-terminal ribonucleotide with an hydroxyl bonded to the 3'-terminal carbon and the 2'-deoxyribonucleotide-5'-phosphate-3'-thiophosphate adduct with group of formula $-OPO_2SCH_2(CO)L_5F_{28}R_{28}$ bonded to the 3'-carbon,

a polynucleotide with group of formula $-OPO_2SCH_2(CO)L_5F_{28}R_{28}$ bonded to the 3'-terminal carbon results. The ligation reaction and subsequent isolation of product is carried out as described by Cosstick et al., supra, in essentially the same way as when 2'-deoxyribonucleoside-5'-diphosphate-3'-thiophosphate is the substrate in the ligation.

The polynucleotide derivatized with $-OPO_2SCH_2(CO)L_5F_{28}R_{28}$ at the 5'-carbon of the 5'-terminal nucleotide or 3'-carbon of the 3'-terminal nucleotide is readily purified chromatographically (e.g., HPLC) prior to use to prepare probe of the invention. If $F_{28}R_{28}$ is $-NH_2$, the preparation of probe from the polynucleotide with derivatized 3'-terminal nucleotide is as described below. If $F_{28}R_{28}$ is $-NHR_{281}$, $NH(C=S)NHR_{282}$ or $-NH(C=O)NHR_{282}$, and probe is not complexed with metal ion, the derivatized polynucleotide is dialyzed against metal-free buffer. If $-F_{28}R_{28}$ is $-NHR_{281}$, $-NH(C=s)NHR_{282}$ or $-NH(C=O)R_{282}$, and probe is complexed with metal ion, the derivatized polynucleotide is subjected to the standard probe chelation process, even if $-R_{28}$ in the substrate for the enzymatic reaction is already complexed with the lanthanide ion, because of the presence of metal ion in the solution required for enzymatic activity of the T4 RNA ligase.

In the foregoing methods, for making polynucleotide with group of formula $-OPO_2S(CH_2)(CO)L_5NH_2$ bonded to the 5'-terminal carbon or the group $-OPO_2S(CH_2)(CO)L_5F_{28}R_{28}$ bonded to the 3'-terminal carbon, it is preferred that L_5 be n-alkyl of 2 to 20 carbon atoms, and most preferred that L_5 be n-alkyl of 4 to 6 carbon atoms.

Is is noteworthy that, by carrying out the above-described modifications at the 5'-terminus of a polynucleotide separately from those at the 3'-terminus, a group of formula $-OPO_2NHL_5NH_2$, $-OPO_2NHL_5SH$ or $-OPO_2SCH_2(CO)L_5NH_2$ can be bonded to the

5'-terminal carbon of the polynucleotide while a group of formula $-OPO_2NHL_{51}NH_2$, $-OPO_2NHL_5SH$ or $-OPO_2SCH_2(CO)L_{51}F_{28}R_{28}$ can also be bonded to the 3'-terminal carbon of the polynucleotide, wherein L_{51} is the same as or different from L_5 and is alkyl of 2 to 20 carbons and wherein the group of formula $-OPO_2NH-$ or $-OPO_2S-$, bonded directly to the 5'-terminal carbon need not be the same as the group, of formula $-OPO_2NH-$ or $-OPO_2S-$, bonded directly to the 3'-terminal carbon.

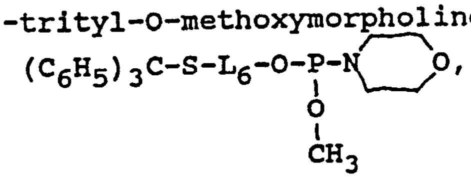
A nucleic acid with a desired sequence and with an amino group ($-NH_2$) bonded to the 5'-terminal carbon is prepared by the method of Smith et al., Nucl. Acids Research 13, 2399-2412 (1985). The method is preferably carried out on an automated synthesizer, such as the Model 380A of Applied Biosystems, Inc. (Foster City, California, U.S.A.). The method of Smith et al. (1985), supra, entails application of the phosphoramidite chemistry of Matteucci and Caruthers, J. Am. Chem. Soc. 103, 3185 (1981), and Beaucage and Caruthers, Tetrahedron Lett. 1981, 1859-1862, to prepare a polynucleotide that is attached to a suitable solid support and that includes the entire sequence of the desired polynucleotide except the 5'-terminal nucleotide. In the method of Smith et al. (1985), supra, a 5'-amino-2'-deoxy-3'-phosphoramidite analog of the desired 5'-nucleotide is prepared, with a suitable protecting group such as trifluoracetyl on the 5'-amino group, and is employed in the final step of the solid phase synthesis. Upon application of known methods in the art, to cleave the polynucleotide from the solid support and deprotect the various protected reactive groups on the cleaved polynucleotide, and known chromatographic procedures to isolate the desired, deprotected polynucleotide, the polynucleotide with the 5'-amino-group on the 5'-terminal nucleotide is obtained.

A nucleic acid with a desired sequence and with a group of formula $-OPO_3(L_6)SSL_5NH_2$, wherein L_5

is alkyl of 2 to 20 atoms, preferably n-alkyl of 2 to 8 carbon atoms, and L_6 is alkyl of 3 to 20 carbon atoms, preferably n-alkyl of 3 to 8 carbon atoms, bonded to the 5′-terminal carbon is prepared in two steps. First, the method of Connolly and Rider, Nucl. Acids Research 13, 4486-4502 (1985), is used to make the nucleotide of the desired sequence and

with a group of formula $-O-\underset{\displaystyle O}{\overset{\displaystyle O}{\overset{\|}{\underset{|}{P}}}}-O-L_6-SH$ bonded to the 5′-terminal carbon. Then, applying well known procedures, the $-OPO_3L_6SH-$ derivatized polynucleotide is reacted with a mixed disulfide of formula $R_5-S-S-L_5-NH_2$, wherein R_5 is 2-pyridyl or 4-pyridyl, to yield the polynucleotide with a group of formula $-OPO_3L_6SSL_5NH_2$ bonded to the 5′-terminal carbon. This polynucleotide is then purified by known chromatographic procedures (e.g., HPLC).

The method of Connolly and Rider also entails application of the phosphoramidite chemistry of Matteucci and Caruthers, supra, and Beaucage and Caruthers, supra, to prepare a polynucleotide that is attached to a suitable solid support and that includes the entire sequence of the desired polynucleotide. Then the protected polynucleotide, attached to solid support, is reacted with an excess of a mercaptoethanol derivative of S-trityl-O-methoxymorpholino-phosphite of formula



wherein L_6 is alkyl of 3 to 20 carbons, preferably n-alkyl of 3 to 8 carbons, followed by oxidation of the resulting phosphite intermediate by the same known procedure used to oxidize the phosphite intermediates in the course of synthesizing the polynucleotide. These S-trityl phosphite derivatives of mercaptoethanols are known compounds, as taught by Connolly and Rider, supra.

The result is a resin-bound polynucleotide with a group of formula

$$-O\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{|}}{P}}-O-L_6-S-C(C_6H_5)_3$$

bonded to the 5'-terminal carbon. The polynucleotide is treated with thiophenolate to remove phosphate protecting groups and then ammonia to remove base protecting groups and cleave polynucleotide from the solid support. The polynucleotide, with the S-trityl bond intact, is isolated by HPLC. Then, in the triethylammonium acetate buffer, pH 6.5, in which the polynucleotide is suspended after the HPLC purification, the polynucleotide is treated with a 5-fold molar excess (relative to polynucleotide) of silver nitrate followed, after 30 minutes, with a 7-fold molar excess of dithiothreitol. The treatment with silver ion cleaves the S-trityl bond. The treatment with dithiothreitol is to remove silver ion. After 30 minutes, the precipitated silver salt of dithiothreitol is removed by centrifugation. The desired, derivatized oligonucleotide remains in the supernatant and is isolated and purified from the supernatant by HPLC, and is then reacted with $R_5-S-S-L_6-NH_2$ in a mixture of acetonitrile/water for 16 hours at 23°C, as described above, to finally obtain the desired polynucleotide, derivatized with $-OPO_3L_6SSL_5NH_2$, which is isolated by chromatography over Sephadex G-50.

The description that follows, of methods of the invention for making a probe of the invention starting with a nucleic acid with the sequence of the probe and with the 5'-terminal nucleotide modified to have a group of formula $-OPO_2(NH)L_5NH_2$ bonded to the 5'-carbon, applies as well to the methods which employ as starting material a nucleic acid with the sequence of the probe and with a group of formula $-OPO_2(NH)L_5NH_2$ bonded to the 3'-terminal carbon, a group of formula $-OPO_2^{-}SCH_2(CO)L_5NH_2$ bonded to the 5'-terminal

carbon or the 3'-terminal carbon, a group of formula $-OPO_2(NH)L_5SH$ bonded to the 5'-terminal carbon or the 3'-terminal carbon, or a group of formula $-OPO_3L_6SSL_5NH_2$ bonded to the 5'-terminal carbon. Although in the description that follows, reference will be limited to the preferred $-OPO_2(NH)L_5NH_2$ group bonded to the preferred position, the 5'-carbon of the 5'-terminal nucleotide, it is to be understood to apply to nucleic acids modified in other ways, as indicated above in this paragraph.

In one method of the invention for making a probe of the invention, a nucleic acid with the sequence of probe and with an amino group ($-NH_2$), or a group of formula $-OPO_2(NH)L_5NH_2$, wherein L_5 is alkyl of 2 to 20 carbon atoms (perferably n-alkyl of 2 to 8 carbon atoms in the case of $-OPO_2(NH)L_5NH_2$ and 2 to 6 carbon atoms in the case of $-OPO_2(NH)L_5SH$), bonded to the 5'-terminal carbon, is reacted in aqueous solution buffered to a pH between about 8 and 10, with an excess, preferably about 20-fold to about 50-fold molar excess relative to concentration of nucleic acid, of PITCP-EDTA, PITCB-EDTA, PICB-EDTA or PICP-EDTA. The reaction is continued for 10 min. to 24 hours, preferably about 4 hours, at between about 0°C and about 40°C, preferably about 4°C. After the reaction, the probe is purified from the reaction mixture by gel permeation chromatography, as, for example, on Sephadex G-50, using a buffer such as 0.01 M Tris-HCl at a pH between about 7 and about 8, as eluant; the standard probe chelation process is then used to complex Eu^{+3}, Tb^{+3} or Sm^{+3} to the probe when desired. The reaction is optionally, and preferably, carried out with the PITCP-EDTA, PITCB-EDTA, PICB-EDTA or PICP-EDTA complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}; if the reaction is so carried out, the eluant in the gel permeation chromatography purification will preferably contain about 0.1 M to 0.5 M sodium citrate and be at pH 6.5 to 7.

If the reactant nucleic acid had an $-NH_2$ group bonded to the 5′-terminal carbon, the probe of the invention resulting from reaction with PITCP-EDTA will have a group of formula -NH(C=S)NH-(p-EDTA-phenyl) bound to said 5′-carbon, and the probe of the invention resulting from reaction with PICP-EDTA will have a group of formula -NH(C=O)NH-(p-EDTA-phenyl) bound to said 5′-carbon, the probe of the invention resulting from reaction with PITCB-EDTA will hae a group of formula -NH(C=S)NH-(p-EDTA-benzyl) bound to said 5′-carbon, and the probe of the invention resulting from reaction with PICB-EDTA will have a group of formula -NH(C=O)NH-(p-EDTA-benzyl) bound to said 5′-carbon. Similarly, if the reactant nucleic acid had an $-OPO_2(NH)L_5NH_2$ group bonded to the 5′-terminal carbon, the probe of the invention resulting from reaction with PITCP-EDTA will have a group of formula $-OPO_2(NH)L_5NH$(C=S)NH(p-EDTA-phenyl) bound to said 5′-carbon, the probe of the invention resulting from reaction with PICP-EDTA will have a group of formula $-OPO_2(NH)L_5NH$(C=O)NH(p-EDTA-phenyl) bound to said 5′-carbon, the probe of the invention resulting from reaction with PITCB-EDTA will have a group of formula $-OPO_2(NH)L_5NH$(C=S)NH-(p-EDTA-benzyl) bound to said 5′-carbon, and the probe of the invention resulting from reaction with PICB-EDTA will have a group of formula $-OPO_2(NH)L_5NH$(C=O)NH-(p-EDTA-benzyl) bound to said 5′-carbon. Similarly, if the group bonded to the 5′ or 3′-terminal carbon was of formula $-OPO_2(NH)L_5SH$, the group, after reaction with PITCP-EDTA, will be of formula $-OPO_2(NH)L_5S$(C=S)NH(p-EDTA-phenyl); after reaction with PICP-EDTA will be of formula $-OPO_2(NH)L_5S$(C=O)NH(p-EDTA-phenyl); after reaction with PITCB-EDTA will be of formula $-OPO_2(NH)L_5S$(C=S)NH(p-EDTA-benzyl); and after reaction with PICB-EDTA will be of formula $-OPO_2(NH)L_5S$(C=O)NH(p-EDTA-benzyl).

If the PITCP-EDTA, PICP-EDTA, PITCB-EDTA or PICB-EDTA reactant is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, the probe resulting from reaction of said reagent with a nucleic acid with the sequence of probe and with the 5'-terminal carbon bonded to an amino group or a group of formula $-OPO_2(NH)L_5NH_2$ will have, linked to said 5'-carbon as indicated above, a p-EDTA-phenyl or p-EDTA-benzyl group that is complexed with said Eu^{+3}, Tb^{+3} or Sm^{+3}.

Chu and Orgel (1985), supra, disclose the synthesis of nucleic acid wherein a group of formula $-OPO_2(NH)(CH_2)_2(NH)R_6$, wherein R_6 is DPTAyl or EDTAyl, is bonded to the 5'-terminal carbon by reaction of nucleic acid, with a group of formula $-OPO_2(NH)(CH_2)_2NH_2$ bonded to said 5'-carbon, with DTPA anhydride or EDTA anhydride respectively. Chu and Orgel (1985), supra, after said synthesis of the DTPAyl or EDTAyl-derivatized nucleic acid, combine it with a solution of Fe^{+2}, and thereby convert the DTPAyl or EDTAyl groups on the nucleic acid to chelates with Fe^{+2}. See also Dreyer and Dervan, supra.

We have now discovered that a nucleic acid with a group of formula $-OPO_2(NH)L_5NH_2$ bonded to the 5'-carbon of the 5'-terminal nucleotide will react with excess EDTA or DTPA, either free or, if DTPA, complexed with a metal ion such as Eu^{+3}, Tb^{+3} or Sm^{+3}, in the presence of excess (relative to EDTA or DTPA) water soluble carbodiimide coupling agent, such as 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide or the preferred 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, to form the probe of the invention, with the group $-OPO_2(NH)L_5(NH)R_6$, wherein R_6 is EDTAyl or DTPAyl, optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, bonded to the 5'-terminal carbon.

Thus, another method of the invention for making a probe of the invention, illustrated in Example III, is to react a nucleic acid, with a sequence of the probe and

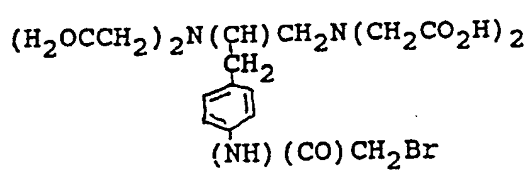
with a group of formula $-OPO_2(NH)L_5NH_2$, wherein L_5 is alkyl of 2 to 20 carbon atoms (preferably n-alkyl of 2 to 8 carbon atoms) bonded to the 5'-terminal carbon, with EDTA, or DTPA (optionally (and preferably) complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}), in aqueous solution buffered to about pH 6 in the presence of a water soluble carbodiimide coupling agent. The preferred reactant is DTPA complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}. The resulting probe can be purified by standard techniques, e.g., chromatographically. If the probe was made with DTPA that was complexed with lanthanide ion, the probe is preferably isolated chromatographically employing 0.1 M to 0.5 M sodium citrate, pH 6.5 to 7, as the eluant. Again, following the same procedure described above for probes made by reacting PITCP-EDTA, PICP-EDTA, PITCB-EDTA or PICB-EDTA with polynucleotide with $-OPO_2(NH)L_5NH_2$ bonded to 5'-terminal carbon, if the probe to be made in this process is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, but the EDTA or DTPA reactant is not, the probe of the invention, with EDTA or DTPA uncomplexed with lanthanide ion linked to the 5'-terminal carbon, is subjected to the standard probe chelation process.

Still another method of the invention for making a probe of the invention comprises providing a nucleic acid, with the sequence of the probe and with an amino group, of formula $-NH_2$, bonded to the 5'-carbon of the 5'-terminal nucleotide, and reacting said nucleic acid with EDTA anhydride or DTPA anhydride at a pH between 6.0 and 8.0. The reaction is carried out with a large molar excess of the anhydride (e.g., 10-10,000-fold over oligonucleotide concentration with reaction volume being adjusted such that the anhydride is at a concentration of 10 mg/ml) and is carried out for about 10 minutes to about 2 hours at room temperature. A typical pH is 7.0, maintained with 0.1 M HEPES. The product probe of the invention is separated from

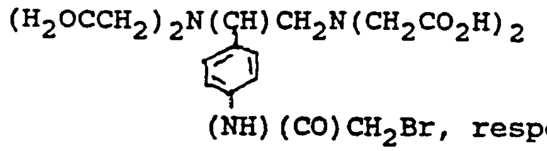
reactants chromatographically, as by HLPC. If the desired probe is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, the probe with EDTAyl or DTPAyl bound through an amide linkage to the 5'-carbon of the 5'-terminal nucleotide is treated by the standard probe chelation process.

A polynucleotide with the sequence of a probe and with $-NH_2$ bonded to the 5'-terminal carbon can also be reacted, in the same way as polynucleotide with a group of formula $-OPO_2(NH)L_5NH_2$ bonded to the 5'-terminal carbon, as described above, with EDTA, or DTPA (optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}), in the presence of a water soluble carbodiimide coupling reagent, to make a probe of the invention. Thus, yet another method of the invention to make probe of the invention comprises providing a nucleic acid with the sequence of the probe and with $-NH_2$ bonded to the 5'-terminal carbon and reacting said nucleic acid, in aqueous solution at a pH of about 6 in the presence of a water soluble carbodiimide coupling agent, with EDTA, or DTPA (optionally and preferably complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}). The resulting probe is isolated chromatographically or by being subjected to the standard probe chelation process, in the same way as probes made with nucleic acid with group of formula $-OPO_2NHL_5NH_2$ bonded to the 5'-terminal carbon. Again, DTPA complexed with lanthanide III ion is the preferred reactant.

A probe of the invention can also be made, employing another method of the invention, by reacting a nucleic acid with the sequence of the probe and with a group of formula $-OPO_2(NH)L_5SH$ bound to the 5' (or 3') terminal carbon with the bromoacetamide derivative of p-EDTA-benzyl or p-EDTA-phenyl of formulae



and



respectively,

wherein the EDTA is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}. Most preferably, the bromoacetamide derivative of p-EDTA-benzyl complexed with Eu^{+3} is employed. The bromoacetamide derivative of p-EDTA-benzyl is known and can be prepared according to Meares et al. Anal. Biochem 142, 68-78 (1984). The bromoacetamide derivative of p-EDTA-phenyl is prepared in the same way as the derivative of the p-EDTA-benzyl except that p-aminophenyl-EDTA is used as the starting material in place of p-aminobenzyl-EDTA. Once prepared, the bromoacetamide derivative is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3} in the same way as PITCP-EDTA, PICP-EDTA, PITCB-EDTA or PICB-EDTA, as described above. Finally, the bromoacetamide derivative complexed with lanthanide ion is taken up in 10 mM HEPES buffer (pH 7.7 and gassed with argon) to a concentration of 1 mM. A 100 ul aliquot of nucleic acid with sequence of probe, derivatized at the 5' (or 3') terminal carbon with a group of formula $-OPO_2(NH)L_5SH$, and stored in 0.01 M DTT, 10 mM HEPES, pH 7.7, as described above, is removed from storage; quickly spin-columned following Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1982), pp. 466-467, using Sephadex-G50 prepared with 10 mM HEPES (pH 7.7) buffer prepared with degassed and deionized distilled water scrubbed to be iron-free; and then combined with 20 ul of the solution of the bromoacetamide derivative in 10 mM HEPES. The pH of the resulting solution is raised to 8.5 with 1 M NaOH and the solution is incubated at 35°C to 40°C for 2 - 6 hours. The resulting probe, complexed with lanthanide ion, is then isolated from the unreacted bromoacetamide derivative chromatographically employing in the eluant 0.25 M sodium citrate, pH 6.8. In the probe, the linker between the 5' (or 3') terminal carbon and the p-EDTA-benzyl or p-EDTA-phenyl complexed with lanthanide-III ion has the formula

$$-OPO_2(NH)L_5SCH_2(CO)(NH)-.$$

A polynucleotide with the sequence of a probe (or probe precursor, if subsequent modification to make probe entails addition of nucleotides) can be prepared by any of several, well known, stepwise solid-phase techniques, such as that of Matteucci and Caruthers, supra, and Beaucage and Caruthers, supra, based on phosphoramidite chemistry, followed by HPLC isolation of the desired nucleic acid. The synthesis can advantageously be carried out with an automated synthesizer, such as the Model 380A of Applied Biosystems, Inc. Significant quantitites of pure, single-stranded polynucleotides of defined sequence up to about 100 nucleotides in length can be prepared by automated, stepwise, solid-phase techniques followed by HPLC purification. The polynucleotides obtained from the automated synthesizer will have hydroxyl group bonded to the 3'-terminal carbon and, consequently, will be suitable as precursors of probes of the invention made by TdT-catalyzed strand extensions or, if the 3'-terminal nucleotide is a ribonucleotide, T4 RNA ligase-catalyzed ligations as described above.

A single-stranded DNA with sequence of probe can also be prepared by cloning into the RF-DNA of a filamentous bacteriophage, such as one of the M13 series (e.g., M13mp18 or M13mp19), a double-stranded DNA which comprises a probing sequence desired for the probe, and then isolating the single-stranded circular DNA genome from phage produced by host bacteria (e.g., E. coli JM103 in the case of phage of the M13 series) transformed with the RF-DNA which includes the double-stranded DNA with probing sequence. The single-stranded phage DNA can be randomly cleaved, as by sonication or with DNAse I (e.g., from bovine pancreas), to a convenient average size, preferably larger than the probing sequence, to provide DNA, with sequence of probe and with 5'-terminal or 3'-terminal phosphate groups, which can be employed, as described above, to make probe of the invention. If cleavage is with DNAse I, only the 5'-terminal nucleotide will be phosphorylated.

Phage DNA fragments with the 3'-carbon of the 3'-terminal nucleotide hydroxylated can be employed as described above, as precursors to make a probe of the invention enzymatically with TdT or, after addition of a 3'-terminal, 3'-hydroxylated ribonucleotide using TdT, T4 RNA ligase.

A double-stranded DNA, which comprises a suitable sequence (e.g., a probing sequence for a target DNA or RNA), can be employed as a source of single-stranded DNA with sequence of a probe of the invention (or a precursor thereof), for modification by methods described above to make probe of the invention. Such double-stranded DNA can also be used as a template for making a DNA or RNA probe of the invention (or precursor thereof) enzymatically, with DNA-dependent DNA polymerase, DNA-dependent RNA polymerase or TdT, as described above. Of course, if DNAse I is employed in combination with the DNA polymerase, the above-described nick-translation method can be applied, using the double-stranded DNA as template, to make probe of the invention (or precursor thereof) (actually a mixture of probes or precursors, due to random cleavage of the double-stranded DNA template by the DNAse I).

A double-stranded DNA which comprises a desired sequence (e.g., a probing sequence) can be prepared by solid-phase, stepwise synthesis of each of the strands, followed by combining them in a solution for annealing into double-stranded form. Alternatively, applying standard cloning procedures, a double-stranded DNA which comprises a sequence, such as a probing sequence, can be cloned in a suitable cloning vector (e.g., plasmid pBR322), and the cloned vector itself can be employed as DNA with sequence of probe or a portion of the vector can be excised, as by digestion of the vector with a suitable restriction endonuclease, and purified, as by agarose gel electrophoresis or any other technique suitable for separating DNAs on the basis of size, and used as DNA with sequence of probe or as a precursor of such DNA.

Most restriction endonucleases leave hydroxylated 3'-carbons on the 3'-terminal nucleotides of each strand of a double-stranded DNA cut by the endonuclease and can thus be employed to provide, from a cloning vector as indicated in this paragraph, a double-stranded DNA that can be used with TdT, as described above, to make probe of the invention or a precursor for such.

The probes of the invention are employed in nucleic acid hybridization assays of samples for the presence of target DNA or RNA, and, consequently, the biological entity uniquely associated with the target DNA or RNA in samples being tested. The probes of the invention are used in such hyridization assays, employing standard techniques for hybridizing probe nucleic acid to target nucleic acid, as follows:

First, nucleic acid is isolated from a sample to be assayed, and is affixed in single-stranded form, to a solid or macroporous support. This procedure is carried out so that a substantial fraction (preferably most) of the target sequence for probe on the target DNA or RNA that might be present in the sample remains intact.

A number of different types of solid support, and methods of affixing sample nucleic acid thereto, can be employed. For example, using procedures well known in the art, nitrocellulose paper can be used. See, e.g., Grunstein and Hogness, supra; Meinkoth and Wahl, supra. Alternatively, the nucleic acid from samples can be affixed covalently by known methods directly to solid beads, such as beads of fine-grained cellulose or Sephadex™, or "beads" of macroporous materials such as agarose (e.g., Sepharose™ or Sephacryl™, such as Sephacryl S-500) See, e.g., Bunemann et al., Nucl. Acids Res. 10, 7163-7180 (1982); Bunemann and Westhoff, Meth. of Enzymol. 100, 401-407 (1983).

By still another method, which is part of the so-called "sandwich hybridization" assay technique, examples of which are also known in the art, a solid or

macroporous support can be provided which has bound to it a first nucleic acid, said first nucleic acid including a probing segment with a sequence that is complementary to the sequence of a first target segment in target nucleic acid. After binding the first nucleic acid to the solid support, and then pre-hybridizing the support, hybridization is carried out with single-stranded nucleic acid of the sample. As a result, target nucleic acid in the sample, if any, becomes affixed to the solid support by base-pairing between the first target segment and the probing segment of said first nucleic acid bound to the support. A second target segment of target nucleic acid, that does not overlap the first target segment, is the target segment for probe of the invention.

In Example VIII, a macroporous-support-first nucleic acid system, and methodology for making and using same, are described. We have found that use of anionic polymer, such as preferably about 10% (w/w) dextran sulfate, in hybridization of sample nucleic acid to macroporous support-bound first nucleic acid and subsequent hybridization of probe to bound sample nucleic acid, substantially improves sensitivity of sandwich assay systems. We have found further that a sandwich assay system, in which the macroporous support-bound first nucleic acid is an oligonucleotide shorter than about 100 bases and the probe is also an oligonucleotide shorter than about 100 bases, is most suitably employed to assay for a target nucleic acid that is single-stranded, such as the RNA genome of certain pathogenic viruses, e.g., HIV-1 virus, which is the causative agent of acquired immune deficiency syndrome.

Next, after nucleic acid from sample has been affixed to the support, the support is pre-hybridized in order to substantially eliminate sites on the support for non-specific binding by probe nucleic acid. As indicated in the foregoing description of affixing target nucleic acid to support when the sandwich hybridization technique

is employed, this pre-hybridization step will have already taken place prior to hybridization between nucleic acid from the sample and the first nucleic acid bound to the support. Thus, with the sandwich hybridization technique, pre-hybridization of support is not needed after nucleic acid from the sample is affixed; but, preferably, in place of this prehybridization, the support will be washed once or twice in a wash procedure (substantially the same as the post-hybridization, high stringency, wash procedure described below) to eliminate from the support nucleic acid from sample that has not stably hybridized to the first nucleic acid bound to the support.

Then, after the pre-hybridization or washing, the support is exposed to a hybridization solution which contains probe of the invention at a molar concentration 10^1-10^{12} times, typically 10^3 to 10^6 times, that of target nucleic acid expected to be on the support, if the sample being analyzed included target nucleic acid. The hybridization is continued for a time period sufficient for formation of duplex between probe and at least a portion (preferably most) of any target nucleic acid segment on the support.

Next, unduplexed or partially duplexed probe is removed from the support by a series of post-hybridization washes, usually 1 or 2, under stringency conditions that ensure that only probe that is stably duplexed to target segment remains in the system and that probe involved in non-homologous heteroduplexes (with nucleic acid segments other than target segment of the probe) is removed from the system.

Those of skill in the nucleic acid hybridization art will understand how to determine readily conditions for attachment of sample nucleic acid to solid or macroporous support, pre-hybridization of the support, and hybridization(s) and post-hybridization washes to ensure the specificity of, and achieve acceptable

sensitivity for, a particular probe of the invention for a particular target nucleic acid segment in samples to be assayed with the probe. See, e.g., Meinkoth and Wahl (1984), supra.

Probe employed in the hybridization solution is preferably complexed, through EDTAyl, DTPAyl, p-EDTA-benzyl or p-EDTA-phenyl group (or groups) chemically linked to it, to Eu^{+3}, Tb^{+3} or Sm^{+3}, most preferably Eu^{+3}.

Finally, probe present on the support, reflecting the presence of target DNA or RNA of the probe in the sample being assayed and the presence in the material from which the sample was obtained of the biological entity associated with said target DNA or RNA, is detected by excitation of fluorescence from the Eu^{+3}, Tb^{+3} or Sm^{+3} complexed with the probe and observation of the resulting fluorescence (i.e., fluorescence emission).

Such fluorescence, from an EDTAyl, DTPAyl p-EDTA-benzyl or p-EDTA-phenyl chelate of Eu^{+3}, Tb^{+3} or Sm^{+3} in an aqueous environment, wherein only water and the EDTAyl, DTPAyl, p-EDTA-benzyl or p-EDTA-phenyl will be involved in the chelation, is relatively weak and short-lived. Thus, sensitivity of a probe involving such a chelate and detected by fluorescence is relatively low and not amenable to enhancement by time-resolved fluorometry. Nonetheless, in assays where a probe of low sensitivity is acceptable, fluorescence can be measured directly from the support with probe bound to chelates of Eu^{+3}, Tb^{+3} or Sm^{+3}, wherein essentially only EDTAyl, DTPAyl, p-EDTA-benzyl or p-EDTA-phenyl group and water molecules are complexed with the lanthanide ion. Because the phenyl group enhances the fluorescence emission of the lanthanide ion, p-EDTA-phenyl or p-EDTA-benzyl are the preferred chelating agent-tag moieties in probes to be detected by fluorescence directly from the tag moiety/water chelate of the Eu^{+3}, Tb^{+3} or Sm^{+3} bound to probe.

The skilled will understand that a hybridization assay of a sample will be conducted in parallel with a hybridization assay of a negative control, which is a sample similar to the test sample but known to be free of target nucleic acid of probe employed in the hybridization assay, and perferably also a hybridization assay of a positive control, which is a sample similar to the test sample but known to include target nucleic acid of the probe used in the hybridization assay. The assays of test sample, negative control and positive control will be run with the same reagents and procedures and at the same time. Then signal (fluorescence emission) from the sample and controls will be compared. A positive signal from positive control establishes that the assay procedures are operative. A signal from test sample that is greater than that from negative control, when the assay procedures are operative, establishes that target nucleic acid is present in the test sample and the associated biological entity is present in the material from which the test sample was prepared. By employing one or more positive controls which include known quantities of target nucleic acid, comparison of fluorescence intensity from a test sample with fluorescense intensity from the negative and positive controls can be used to estimate the amount of target nucleic acid in the test sample and the titer of the associated biological entity in the material from which the test sample was prepared.

The preferred method for detecting probe is to proceed as follows:

First, the support, with probe-lanthanide ion complex bound (if target nucleic acid of probe was in the sample being assayed), is incubated with an "enhancement solution." Then fluorescence of the resulting solution (which will include lanthanide ion chelates in micelles if probe-lanthanide ion complex was bound to the support) is measured directly with excitation and observation of

emission at wavelengths characteristic of the lanthanide ion involved. The preferred lanthanide ion is Eu^{+3}. Preferably time-resolved fluorometry is employed, using any of numerous devices for measurement of time-resolved fluorescence that are commercially available. However, regular fluorescence (i.e., not time-resolved), using a standard fluorescence spectrometer, and even simple visual inspection of the solution for color characteristic of fluorescence from the lanthanide ion, when the solution is irradiated with light capable of exciting the fluorescence, can be employed, particularly in applications where extremely high sensitivity is not required.

A typical enhancement solution will be an aqueous solution, will have a pH between 2.8 and 3.5 maintained with a suitable buffer (e.g., phthalate-HCl), typically at about 0.1 M concentration, will include about 0.1% (v/v) to about 0.5% (v/v) of a non-ionic detergent, such as Triton X-100 or a Tween (e.g., Tween-20 or Tween-80), suitable for forming micelles capable of sequestering ß-diketone/Lewis base chelates of lanthanide ion from water, will include between about 10 uM and 100 uM of a ß-diketone, and will include between about 10 uM and about 100 uM of a Lewis base.

The ß-diketone employed in the enhancement solution is of formula $R_{20}(CO)CH_2(CO)CF_3$, wherein R_{20} is 2-naphthyl, 1-naphthyl, 4-fluorophenyl, 4-methoxyphenyl, or phenyl. The most preferred of the ß-diketones is 2-naphthoyltrifluoroacetone.

The Lewis base employed in the enhancement solution is a synergistic (sometimes referred to in the art as "synergic") Lewis base selected from O-phenanthroline, triphenylphospine oxide, or a trialkylphosphine oxide, wherein the alkyl groups are the same or different and are each of 1 to 10 carbon atoms. The most preferred of the Lewis bases is TOPO (tri-n-octylphosphine oxide).

A preferred enhancement solution consists of 0.1 M phthalate-HCl buffer, pH 3.2; 20 uM 2-naphthoyltrifluoroacetone, 50 uM TOPO and 0.1% (v/v) Triton X-100.

The enhancement solution is incubated with probe on the support at room temperature for 1 second to 24 hours, preferably about 1 minute, prior to measurement of fluorescence.

The enhancement solution serves to increase the fluorescence of the lanthanide ion, and thereby the sensitivity of probes of the invention, by a multistep process:

1) Because the buffer is of a pH near, or lower than, the pK_a of the carboxyl groups on the polyaminocarboxylate tag moiety-chelator linked to probe (i.e., pH 2.5-4), the tag moiety-chelator is protonated and, thereby, its dissociation constant for lanthanide ion substantially increased, resulting in release of the ion.

2) Once free in solution, the lanthanide ion is chelated by the ß-diketone.

3) The Lewis base may also be a ligand in chelates with the lanthanide ion and increase fluorescence intensity from the ion; but, more significantly, the Lewis base interacts with ß-diketone ligand in such chelates to deprotonate the ß-diketone and thereby enhance fluorescence from the chelates due to the increased delocalization of charge when the ß-diketone is in the anionic form.

4) The detergent forms micelles in which the diketone-lanthanide ion chelates cluster and become effectively shielded from water. Because water quenches fluorescence from lanthanide ion, the clustering in micelles arising from presence of the detergent further enhances fluorescence intensity and also enhances fluorescence lifetime from the lanthanide ion chelates. Enhanced fluorescence lifetime makes possible the use of

time-resolved fluorometry to distinguish fluorescence from lanthanide ion from short-lived background fluorescence (e.g., from non-target nucleic acid and support material to which nucleic acid is affixed) and thereby enhance sensitivity of probes of the invention.

With the preferred lanthanide ion, Eu^{+3}, in an enhancement solution combined with a probe of the invention, fluorescence excitation is at about 340 nm and fluorescence emission is observed at about 613 nm.

Many of the compounds and groups involved in the instant specification (e.g., phosphate, EDTA, amino) have a number of forms, particularly variably protonated forms, in equilibrium with each other. As the skilled will understand, representation herein of one form of a compound or group is intended to include all forms thereof that are in equilibrium with each other.

In the present specification, "uM" means micromolar, "ul" means microliter, and "ug" means microgram.

The invention is now further described and illustrated in the following examples:

EXAMPLE I

Preparation of Cyclic Anhydrides of Ethylene Diamine Tetraacetic Acid (EDTA) and Diethylene Triamine Pentaacetic acid (DTPA)

The cycle anhydrides were prepared as described by Hnatowich, et al., Int. J. Appl. Radiat. Isot., 33, 327-332 (1982).

To 3.93 g (0.01 moles) of DPTA was added 5 ml of dry pyridine and 3 ml (0.04 moles) of acetic anhydride. The mixture was heated for 23 hours at 65°C under an argon atmosphere. The resulting mixture was filtered and

the collected solid was washed with four 15 ml portions of anhydrous ether, and then dried for 2 to 16 hours in vacuo. The product was an off-white solid, MP. 176°C (Dec.). Yield: 93%.

The same procedure was followed with EDTA in place of DPTA. The product was obtained in 85% yield and had a melting point of 192°C (Dec.).

EXAMPLE II

(A) Sequences of Probe for Hepatitis B Virus

A 29 base-pair segment of the hepatitis B virus genome has been identified, each strand of which, when employed as DNA with sequence of a probe, provide probes of surprising sensitivity and specificity in hybridization assays for diagnosis of hepatitis B infection. The same is the case for the two 29 base RNA's with the RNA sequences corresponding to the sequences of the two DNA segments. The 29 base-pair segment of the viral genome is:

5'-AACCAACAAGAAGATGAGGCATAGCAGCA-3'
3'-TTGGTTGTTCTTCTACTCCGTATCGTCGT-5'

wherein all of the nucleotides are 2'-deoxyribonucleotides. In the RNA segments, all of the nucleotides are ribonucleotides and T's in the DNA sequence are replaced by U's in RNA sequences.

Another aspect of the instant invention, then, are nucleic acid probes with these four sequences. The probes can be labeled for detection by any tag, including radioactive or chemical, in accordance with labels and labeling methods of the present invention or otherwise. The 29-base nucleic acid segments can be made in large quantities, in highly pure form, by phosphoramidite chemistry carried out on an automated synthesizer,

followed by chromatographic purification, as illustrated in Example III. Also included in the invention are various derivatives of the four segments which at derivatized at the 5'-terminal or 3'-terminal carbons and are intermediates in making probes, including derivatives with the combination of terminal labels indicated as follows:

Moiety bonded to 5'-carbon	Moiety bonded to 3'-carbon
-OH	-OH
$-OPO_3$	-OH
$-NH_2$	-OH
$-O\overset{\overset{S}{\parallel}}{P}O_2$	-OH
$-OPO_3L_6SH$	-OH
-OH	$-OPO_3$
$-OPO_3$	$-OPO_3$

Methods of making these derivatives are well known in the art.

(B) Sequences of Probe for HIV-1 Virus

Polynucleotides with the following sequences (where, if the polynucleotide is an RNA, a "T" represents a uridine) have been found to be useful as probes for the HIV-1 virus:

5'-CAAAAACTATTCTTAAACCTACCAAGCCTC-3'
5'-TATTACATTTTAGAATCGCAAAACCAGCC-3'
5'-TAGGTTTCCCTGAAACATACATATGGTGT-3'
5'-TGGTCTGCTAGTTCAGGGTCTACTTGTGTGC-3'
5'-CACCTAGGGCTAACTATGTGTCCTAATAAGG-3'
5'-TTTCGTAACACTAGGCAAAGGTGGCTTTATC-3'

5'-TGGTCTTCTGGGGCTTGTTCCATCTATCCTC-3'
5'-AGGGAAAATGTCTAACAGCTTCATTCTTAAC-3'
5'-AAATGGATAAACAGCAGTTGTTGCAGAATTC-3'
5'-TCGAGTAACGCCTATTCTGCTATGTCGACAC-3'
5'-CTGTGTAATGACTGAGGTGTTACAACTTGT-3'
5'-TCTAATTACTACCTCTTCTTCTGCTAGACT-3' and
5'-AATATGTTGTTATTACCAATCTAGCAT-3.

Such polynucleotides can also be derivatized in various ways, as indicated above for the probes for hepatitis B virus.

EXAMPLE III

Preparation of Polynucleotide-Chelate Conjugates

A 29-base polynucleotide (DNA) of Example II, of sequence:

5'-AACCAACAAGAAGATGAGGCATAGCAGCA-3'

was prepared on an Applied Biosystems Synthesizer, Model No. 380A (Applied Biosystems, Inc., Foster City, California, U.S.A.) using cyanoethyl phosphoramidite chemistry. Tritylated polynucleotide was purified using C_{18} reverse-phase, semipreparative chromatography (10 x 250mm column), eluting over a period of 40 minutes using a gradient of 15-35% acetonitrile in 0.1M triethylammonium acetate, pH 6.6. Detritylation of the purified polynucleotide was then accomplished by treatment with 80% acetic acid, and the detritylated polynucleotide was then chromatographically purified using G-50 Sephadex™ in 0.2X TE buffer (1XTE buffer is 10 mM Tris-HCl, 1 mM EDTA, pH 8).

If desired, further purification can be accomplished by subjecting the G-50 Sephadex™ purified polynucleotide to high performance liquid chromatography on an RPC-5 column (4.6 x 250mm) using, for solvent A, 2mM Tris, pH12 and, for solvent B, 2mM Tris, 200mM perchlorate, pH12, and a gradient of 10%B to 50%B over 40 minutes.

1.5 ug of the purified polynucleotide was phosphorylated using standard procedures with T4 polynucleotide kinase and ^{32}P-labeled ATP.

300 ng of the hexylene diamine adduct of the phosphorylated polynucleotide was prepared following Chu, B. F., et al. (1983), supra, as follows: 300 ng of the polynucleotide was taken up in 200 ul of 0.1 M methyl imidazole, 0.25 M hexylene diamine pH 6.0 and the reaction was allowed to proceed for 16 hours at 23°C. The adduct was purified from the final reaction mixture by gel permeation chromatography over Sephadex G-50 using 0.05 M HEPES, pH 7.0, as eluant.

The DTPA adduct of the hexylenediamine-derivatized polynucleotide was then prepared as described by Chu and Orgel, Proc. Nat. Acad. Sci. (US), 82, 963 (1985), except DTPA anhydride was used in place of EDTA anhydride:

The 300 ng of hexylenediamine adduct isolated from Sephadex G-50 chromatography was ethanol-precipitated and the resulting pellet was dried. 5 mg of DTPA anyhdride, prepared as in Example I, was added to the dried pellet. To this was added 0.5 ml of 0.1 M HEPES, pH 7.0 and the mixture was vortexed for 5 minutes and allowed to react for a further 55 minutes at 23°C. The oligonucleotide was ethanol-precipitated, followed by purification by gel permeation chromatography using Sephadex G-50 in 0.01 M Tris pH 7.4 and then another ethanol-precipitation. The resulting pellet was taken up in 200 ul of 10 mM $EuCl_3$ solution containing 1 mM phathalate, pH 3.0. After 5 min., the pH was adjusted to 6-7 with NaOH and the mixture was frozen and stored at -20°C until use. Alternatively, and preferably, the pellet is taken up in 200 ul of 0.1 M sodium citrate buffer, pH 6.8, and to this solution, cooled on ice, is added 200 ul of 0.2 M HCl containing 0.2 mM $EuCl_3$. The pH of the resulting solution is adjusted to 3.2 with aqueous NaOH or HCl, as necessary, and the solution is incubated on

ice for 15 minutes. After the 15 minutes, the pH of the solution is adjusted to 7 with 1 M NaOH, and the resulting solution is stored at -20°C until use.

As an alternative procedure, a 1 mM solution of $EuCl_3$ in 0.01 N HCl is prepared in the presence of 1 mM of DTPA. By adjusting the pH from 2 to 6, by the addition of sodium bicarbonate, the DTPA chelate of europium forms. 200 ul of the resulting solution is added to 200 ng of ethylene diamine-derivatized oligonucleotide, prepared as described above for the hexylenediamine adduct but using ethylene diamine in place of hexylenediamine, in 150 ul of 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The mixture is allowed to react at pH 6 at room temperature for 24 hours and the desired product is isolated by ethanol-precipitation.

When DTPA, complexed with Eu^{+3}, is used in the coupling to the ethylene diamine-derivatized polynucleotide in the presence of carbodiimide coupling agent, under the conditions of the previous paragraph, essentially the same results are obtained as when DTPA was first coupled and then the DTPA-coupled polynucleotide was combined with Eu^{+3}.

EXAMPLE IV

Eu^{+3} Chelates of 1-(p-isothiocyanato-phenyl)EDTA and 1-(p-diazo-phenyl)EDTA

1-(p-amino-phenyl)EDTA is prepared as described by Sundberg, et al., J. Med. Chem., 17 1304-1307 (1974).

Then, following Hemmila et al., supra, 10 ml of chloroform is added to the solution of 1-(p-amino-phenyl)EDTA and the mixture is treated with 25 mg of thiophosgene. After rapid stirring for 30 minutes, the aqueous layer is separated and washed three times with chloroform. 1-(p-isothiocyanato-phenyl)-EDTA is isolated from the dried aqueous layer.

The 1-(p-diazo-phenyl)EDTA (PDP-EDTA) is freshly prepared, following the procedure of Sundberg et al., supra, by treating 1-(p-amino-phenyl)EDTA, at about 0.2 M concentration in H_2O, prepared as described above, with $NaNO_2$/HCl, destroying excess $NaNO_2$ by addition of urea, and finally diluting by addition of H_2O to a final volume about 60 to about 70 times that of the solution of 1-(p-amino-phenyl)EDTA used as staring material.

The PITCP-EDTA and PDP-EDTA are chelated with Eu^{+3} as follows: To 10 ml of a 3 mM solution of the PITCP-EDTA in 0.1 M HCl or the solution of PDP-EDTA prepared as just described is added with stirring 11.5 mg $EuCl_3.6H_2O$. Following the addition, the pH is brought to 7 by the addition of solid $NaHCO_3$. The resulting solution is centrifuged to pellet excess europium, which precipitates above pH 6.5, and the supernatant, which is a solution of the desired chelate, is saved.

EXAMPLE V

Labeling of Nucleic Acids with the Europium Chelate of PDP-EDTA

To 1 ml of a solution, prepared as in Example IV, that is about 3 mM in the PDP-EDTA chelate, is added 1 ml of a solution of 10 ug/ml of DNA, isolated from M13mp18 phage, and 0.4 M borate buffer, pH 8. After stirring the resulting solution for 4 hours at 4°C, the labeled probe is purified by gel permeation chromatography on Sephadex G-50 using either 0.2 M sodium citrate, pH 6.8, or a solution of 0.01 M Tris-HCl (pH 7.0), 20 uM DTPA, and 50 uM $CaCl_2$ as eluant.

EXAMPLE VI

Labeling by Nick-Translation of Nucleic Acids with Europium-DTPA Chelate of 5-Allylamine dUTP

1 ug of plasmid pUC19 (purchased from Bethesda Research Laboratories, Gaithersburg, Maryland, U.S.A., Catalog No. 5364SA) is taken up in 5 ul of 0.5 M Tris-HCl (pH 7.2), 0.1 M $MgSO_4$, 1 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin. To this is added 1 nmole of the unlabeled 2'-deoxynucleoside-5'-triphosphates (dATP, dGTP, dCTP) and also 100 pmole of the DTPA-chelate of 5-allylamine-2'-deoxyuridine-5'-triphosphate prepared as follows:

To 1 umole of 5-allylamine dUTP is added 1 ml of a 10 mg/ml solution of DTPA anhydride in 0.2 M HEPES buffer (pH 7.0). After 30 minutes at 23°C the triphosphate-DPTA analog is purified from the reaction mixture by HPLC using a 0.1 M ammonium acetate, pH 6.5, gradient. The triphosphate analog is collected and lyophilized.

The solution of deoxynucleoside triphosphates for nick-translation is brought to 44 ul with water. To this is added 2 ul of E. coli DNA polymerase I (2 units/ml) and 1 ul of a 0.1 ug/ml solution of bovine pancreatic DNAse I. After one hour at 15°C, the mixture is immersed in a 80°C water bath for 10 minutes and then cooled to room temperature. The labeled nucleic acids comprising DTPA-chelate-5-allylamine-2'-deoxyuridines are then separated from nucleoside-5'-triphosphates and nucleoside-5'-triphosphate 5-allylamine analog and purified by chromatography over Sephadex G-50 using 0.01 M Tris (pH 7.4) as eluant.

The DTPA-derivatized nucleic acid is complexed with Eu^{+3} as follows: 200 ng of the nucleic acid is dissolved in 100 ul of a 0.1 M sodium citrate solution, pH 6.7, the solution is cooled on ice and is combined

with 100 ul of a 0.2 M HCl solution with 0.1 uM $EuCl_3$. The pH of the resulting solution is adjusted to pH 3.2 by addition of NaOH or HCl as necessary and is then incubated on ice for 15 minutes. The pH of the solution is then raised to 6.7 by addition of 1 M NaOH. The nucleic acid-Eu^{+3} chelate is isolated by gel permeation chromatography on Sephadex G-50 using a solution of 0.2 M sodium citrate (pH 6.8) as eluant.

EXAMPLE VII

Alternative Preparation by Nick-Translation of Nucleic Acids Labeled with Europium-DTPA Chelate of 5-Allylamind dUTP

The nick-translating procedure of Example VI is followed, except that 100 pmole of 5-allylamine-2'-deoxyuridine-5'-triphosphate is used in place of the DTPA-chelate thereof.

300 ng of the 5-allylamine-derivatized nucleic acid is dissolved in 25 ul of 0.2 M HEPES (pH 7.7) containing 10 mg/ml of DTPA anhydride. The ensuing reaction is continued for 8 hours at room temperature. The DTPA-derivatized nucleic acid is then separated from the allylamine-derivatized by HPLC.

Finally, following the chelation procedure of Example VI, the nucleic acid-Eu^{+3} chelate is obtained.

EXAMPLE VIII

Specificity and Hybridization Efficiency of Lanthanide Ion Chelate-labeled Probe

The DNA ("complementary oligonucleotide") with the sequence complementary to that of the polynucleotide

of Example III (A) and another DNA ("non-complementary oligonucleotide") with the sequence

5'-AATTCACCATGATGTTCTCGGGTTT-3'

were synthesized and purified in the same manner as the polynucleotide of Example III(A).

The complementary oligonucleotide was bound to agarose beads (Sephacryl S-500™ macroporous support, purchased from Pharmacia, Inc., Piscataway, N. J., U.S.A.) as follows:

A volume of Sephacryl-500™ as supplied by Pharmacia was washed five times with an equal volume of distilled water to remove azide. Then the Sephacryl, in the form of a packed gel, was suspended in water (1 ml of Sephacryl in 4 ml total volume) and the suspension was cooled on an ice bath. Then, as the cooled suspension was stirred with an overhead stirrer, cyanogen bromide (0.4 g CNBr per gram suspension) was added. Stirring was continued for 30 minutes with maintenance of pH between 10.5 and 11.5 by addition of 3 M KOH. After the 30 minutes, the resulting suspension was filtered and then washed five times, each with a volume of cold distilled water equal to the volume of "gel" remaining on the filter, and, finally, once with the same volume of cold, 10 mM potassium phosphate buffer pH 8. After the wash with buffer, the "gel" was immediately transferred to a flask, to which was added quickly 6-aminocaproic acid ($NH_2(CH_2)_5CO_2H$) (0.8 g per gram of "gel") and enough 10 mM potassium phosphate buffer (pH 8) to bring the volume to 8 ml per gram of "gel". The resulting mixture was stirred at room temperature for 12 to 24 hours. Then the gel was filtered and the resulting solid was washed with, in the following order, 10 mM potassium phosphate buffer (pH 8), 1 M potassium phosphate buffer (pH 8), 1 M KCl, 0.1 M NaOH, and distilled water. The resulting, aminohexanoic acid-derivatized gel was then stored at 4°C in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4.

Purified complementary oligonucleotide was 5'-phosphorylated with ATP and T4 polynucleotide kinase by a standard technique. The kinased nucleotide (25 ug/ml of kinase reaction solution) was then purified by adding to 0.3 ml of the solution 0.04 ml of 8 M LiCl solution and 0.9 ml absolute ethanol, freezing the resulting solution on dry ice, centrifuging at room temperature for 10-15 minutes to form a pellet, and then withdrawing and discarding supernatant with a pulled pipette. The pellet (approximately 7 ug) of the purified, kinased oligonucleotide was then dissolved in 300 ul of 0.25 M ethylenediamine ("EDA"), 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide ("CDI") and 0.1 M methylimidazole ("MeIm"), pH 6.0, and allowed to react for 16 hours at 23°C. The resulting EDA-derivatized oligonucleotide was then pelleted, after being mixed with LiCl and ethanol and frozen, as described above for the kinased oligonucleotide. Then, to remove any contaminating EDA, the derivatized oligonucleotide was twice taken up in 0.1 M MES buffer, pH 6, and pelleted, with LiCl/ethanol and freezing, as above. The final pellet (approximately 6 ug) was taken up into 300 ul of 0.1 M MES buffer, pH 6.0.

The EDA-derivatized oligonucleotide was then bound to the aminohexanoic acid-derivatized Sephacryl-500 "gel" (i.e., macroporous support) as follows: 50 mg of support was taken from storage, washed with 0.1 M MES, and then taken up in 0.55 ml of 0.1 M CDI and 0.1 M MES buffer, pH 6, in a 1.8 ml Nunc tube. To this suspension was added 25 ul of solution of the EDA-derivatized complementary oligonucleotide (approximately 20 ng/ul) in 0.1 M MES buffer, pH 6. The tube was then put on a Sepco tube rotator for stirring for 16-20 hours at room temperature. The support was then pelleted by centrifugation, and then washed three times, each time by being shaken with 1.5 ml of 0.01 M NaOH, pelleted by centrifugation, and having supernatant removed by

pipette. The support, after the final wash, was suspended until use in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4.

Approximately 0.7 pmole of oligonucleotide was bound per mg of Sephacryl S-500 bead prepared as described.

The non-complementary oligonucleotide was EDA-derivatized and bound to aminohexanoic acid-derivatized Sephacryl S-500 beads by the same procedure as the complementary oligonucleotide and was bound to the same extent, approximately 0.7 pmole/mg.

Hybridizations were then carried out between each of the doubly labeled polynucleotide of Example III (i.e., labeled at the 5'-terminus with both ^{32}P-phosphate and DTPA-Eu^{+3} chelate) and singly labeled polynucleotide of Example III (i.e., labeled at the 5'-terminus only with ^{32}P-phosphate), and each of the complementary oligonucleotide bound to Sephacryl and the non-complementary oligonucleotide bound to Sephacryl. All of the hybridizations were carried out as follows:

A solution of 6 X SSC, 0.1% (w/v) sodium dodecyl sulfate and 10% (w/v) Dextran sulfate (Pharmacia, Inc.) was prepared. "SSC" is standard sodium citrate well known in the art.

A hybridization solution was prepared by combining 750 ul of this SSC/SDS/Dextran sulfate solution with 30 mg of Sephacryl beads with oligonucleotide bound (20 pmole oligonucleotide) and 50 fmole of labeled oligonucleotide. The hybridization solution was incubated for 90 minutes at 23°C. Then the Sephacryl beads were pelleted and washed three times with 2X SSC at 23°C. The quantity of labeled oligonucleotide bound to the beads was determined by measuring radioactive decay of ^{32}P.

Results were as follows:

Labeled Oligonucleotide	Bead-bound Oligonucleotide	Labeled Oligonucleotide Bound to Beads (fmole)
Doubly Labeled	Complementary	24
Doubly Labeled	Non-Complementary	less than 0.5
Singly Labeled	Complementary	30
Singly Labeled	Non-Complementary	less than 0.5

Thus, employing a lanthanide III chelate tag to label a nucleic acid probe does not interfere with the specificity of the probe and does not interfere significantly, if at all, with the hybridization efficiency of the probe.

EXAMPLE IX

Preparation of Lanthanide Fluorescence Enhancers and Detection Components

2-Napthoyltrifluoroacetone was prepared by a modification of the method of Reid and Calvin (J. Amer. Chem. Soc. 72, 2948-2949 (1950)), as follows: To 10.5 mmoles of sodium methoxide was added 20 ml of dry benzene under a nitrogen atmosphere. 10 mmoles of S-ethylthiotrifluoroacetate was added followed by 10 mmoles of 2-napthyl methyl ketone. After stirring for 20 hours at 20°C, the reaction mixture was dried under reduced pressure. The solid was washed with 100 ml of 10% sulfuric acid and the organic layer was washed with 100 ml of water and dried under reduced pressure. Pure 2-napthoyltrifluoroacetone was crystallized from ethanol/water. 2.31 g (44%) of a fluffy, fluorescent white powder was isolated. M.P. 67-69°C (Lit. 70-71°C).

The fluorescence enhancement solution was prepared according to the method of Hemmila et al., Anal. Biochem., 137, 335-343 (1984). The buffer was composed

of 0.1 M phthalate (pH 3.2) containing 15 uM 2-napthoyltrifluoroacetone, 50 uM tri-n-octylphosphine oxide, and 0.1% (v/v) Triton X-100.

EXAMPLE X

Detection of Hybridized Eu^{+3} Chelate-Tagged Nucleic Acid Probes by Visual Observation of Fluorescence

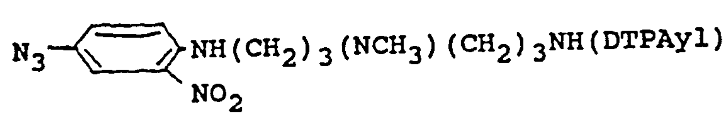
500 ul of the fluorescence enhancer buffer from Example IX was added to 30 mg of each of the four bead-probe combinations prepared as in Example VIII, after hybridizations and washes as described in Example VIII. After 5 minutes incubation, the samples were illuminated with an ordinary ultraviolet lamp and visually inspected. The sample with doubly-labeled probe hybridized to complementary oligonucleotide was dark red. The sample with doubly-labeled probe hybridized to non-complementary oligonucleotide was faintly red. The other two samples remained clear.

EXAMPLE XI

Phenyl Azide-Derivatized DTPA or EDTA

The synthesis of the title compounds is essentially as described by Fleet et al. Biochem. J. 128, 499-508 (1972) and Forster et al., supra.

The DTPA derivative of formula



was prepared as follows:

To 5 g of 4-fluoro-3-nitroaniline (Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.) was added 30 ml of concentrated HCl and 5 ml of water. This was cooled to -20. 2.4 g of $NaNO_2$ was dissolved in 5 ml of water and this was added to the above solution dropwise in order to keep the temperature below -15. All subsequent reactions were carried out in the dark. After 15 minutes, the solution was filtered and to the filtrate was added 2.2 g of NaN_3 in 8 ml of water. The tan precipitate was collected and washed with several portions of cold water. The solid was dissolved in hot hexane which yielded 5.04 g (84%) of the desired 4-fluoro-3-nitrophenyl azide (MP 52-53°C, homogeneous as determined by thin layer chromatography (TLC)) as yellow orange needles.

To 1.8 g of 4-fluoro-3-nitrophenyl azide in 20 ml of ether was added dropwise a solution of 6.4 ml of 3,3'-diamino-N-methyldipropylamine [formula: $H_2N(CH_2)_3(NCH_3)(CH_2)_3NH_2$] in 40 ml of ether. (Note: Any diamine compound can be used in this reaction step to generate an amine-terminated phenyl azide.) After 6 hours the ether was removed and the solid residue was dissolved in chloroform. The desired compound was isolated by flash chromatography over silica gel using 10% methanol in chloroform. 3.0 g (98%) of a deep red oil was isolated. The compound identity was confirmed by TLC, NMR, and IR.

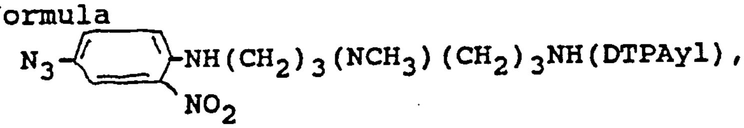
To 307 mg of the above phenyl azide-amine was added 500 mg of DTPA anhydride in a solution of 20 ml of N,N'-dimethylformamide containing a drop of triethylamine. After 2 hours, the solution was concentrated and the compound purified by partition chromatography using the two phase solvent system generated by mixing 1-butanol, acetic acid, and water in 4:1:4 volumetric portions. The main red peak was pooled and dried to yield the desired photoactive, phenyl azide-derivatized DTPA as a thick red oil.

EXAMPLE XII

Use of Phenyl Azide-Derivatized DTPA or EDTA to Label Nucleic Acid with Lanthanide III Ion

In this example, the phenyl azide-derivatized DTPA of Example XI is employed to illustrate the use of phenyl azide-derivatized DTPAs and EDTAs of the invention to label nucleic acids non-specifically with lanthanide III ion.

A stock solution at 1 mg/ml in water was prepared with the phenyl azide-derivatized DTPA of formula



prepared as in Example XI. The solution was prepared in the dark and stored in the dark at -20°C.

The phenyl azide-derivatized compound is chelated in the dark with Eu^{+3} as follows: To 5 ml of the approximately 1.5 mM stock solution is added 0.5 ml of 1 M HCl and then, with stirring, 2.9 mg of $EuCl_3.6H_2O$. Following the addition of the $EuCl_3$, the pH is brought to 7 by the addition of solid $NaHCO_3$. The resulting solution is centrifuged to pellet excess europium and the supernatant, which is a solution of the desired chelate at about 1.3 mM concentration, is saved.

Again in the dark, to a silanized 13 X 50 mm glass test tube is added 5 ug of single-stranded DNA from M13mp18 phage in 25 ul of 0.2 M sodium citrate buffer, pH 7, and 6 ul of the above-described solution of approximately 1.3 mM Eu^{+3} chelate of phenyl azide-derivatized DTPA. The volume is adjusted to 50 ul with H_2O. The resulting solution is placed in an ice bath and photolyzed with a standard laboratory 250 watt "white light" lamp (General Electric Co.) for 30 minutes with the light bulb approximately 10 cm from the solution

at point of closest approach. The resulting, Eu^{+3}-labeled probe is purified by gel permeation chromatography on Sephadex G-50 using 0.2 M sodium citrate, pH 6.8, as eluant.

The foregoing examples illustrate the present invention, but are not intended to limit the scope of the invention. Those skilled in the art will recognize modifications and variations of the exemplified embodiments that are within the spirit and scope of the invention described and claimed herein.

WHAT WE CLAIM IS:

1. A nucleic acid probe comprising a tag moiety selected from the group consisting of EDTAyl, DTPAyl, p-EDTA-phenyl and p-EDTA-benzyl, said tag moiety:

(A) linked to the nucleic acid of the probe by a linker moiety that is terminated, at the bond with the tag moiety, with a group of a formula selected from the group consisting of -NH-, -NH(C=S)NH-, -NH(C=O)NH-, -S(C=S)NH-, -S(C=O)NH, or $-S(CH_2)(CO)(NH)-$ provided that, if said terminal group of the linker moiety is -NH(C=S)NH-, -NH(C=O)NH-, -S(C=S)NH, -S(C=O)NH-, or $-S(CH_2)(CO)(NH)-$ the tag moiety is p-EDTA-phenyl or p-EDTA-benzyl;

(B) linked through said linker moiety to a nucleoside base, the 5'-terminal carbon or the 3'-terminal carbon of the nucleic acid of the probe; and

(C) optionally complexed with a lanthanide-III ion selected from the group consisting of Eu^{+3}, Tb^{+3} and Sm^{+3}.

2. A nucleic acid probe according to Claim 1 wherein the tag moiety is EDTAyl or DTPAyl.

3. A nucleic acid probe according to Claim 2 wherein the tag moiety is complexed with Eu^{+3}.

4. A nucleic acid probe according to Claim 3 wherein the nucleic acid is at least 12 and not more than 100 nucleotides in length.

5. A nucleic acid probe according to Claim 1 comprising:

(A) a uracil or cytosine moiety bonded through carbon-5 to a group of formula $-F_{35}L_{35}F_{20}R_{10}$,

(B) a cytosine moiety bonded through the N^4-nitrogen to a group of formula $-F_{36}L_{36}F_{20}R_{10}$,

(C) a guanine or adenine moiety bonded through carbon-8 to a group of formula $-F_{38}L_{38}F_{20}R_{10}$; wherein $-F_{35}-$ is -CH=CH-, -CH=CH(CO)(NH)-, $-(CH_2)_2(CO)(NH)-$, or $-CH=CHCH_2NH(CO)_a-$, wherein -CH= or $(CH_2)_2$ is bonded to carbon-5 and wherein a is 0 or 1; wherein, when $F_{35}-$ is -CH=CH-, -CH=CH(CO)(NH)-, $-(CH_2)_2(CO)(NH)-$ or a group terminated with a carbonyl group, L_{35} is n-alkyl of 1 to 20 carbon atoms, $-L_{351}(NH)(CO)L_{352}-$ or $-L_{351}(CO)(NH)L_{352}-$, wherein $-L_{351}-$ is n-alkyl of 1 to 17 carbon atoms and is bonded to $-F_{35}-$ and wherein $-L_{352}-$ is alkyl of 1 to 17 carbon atoms, provided that $-L_{351}-$ and $-L_{352}-$ together have no more than 18 carbon atoms; wherein, when $-F_{35}-$ is terminated with an amino group, L_{35} is $-CH_2(CHOH)CH_2O(CH_2)_bOCH_2(CHOH)CH_2-$, wherein b is 2 to 20;

wherein $-F_{36}-$ is -NH-, -NH(C=S)NH-, -NH(C=O)NH-, or $-N=C(R_{33})-$, wherein the nitrogen is bonded to the N^4-nitrogen and R_{33} is hydrogen or alkyl of 1 to 4 carbon atoms;

wherein $-L_{36}-$ is alkyl of 2 to 20 carbon atoms;

wherein $-F_{38}-$ is O, S or -NH-;

wherein $-L_{38}-$ is n-alkyl of 2 to 20 carbon atoms, $-L_{381}(NH)(CO)L_{382}-$ or $-L_{381}(CO)(NH)L_{382}-$, wherein $-L_{381}-$ is n-alkyl of 1 to 17 carbon atoms and is bonded to $-F_{38}-$ and $-L_{382}-$ is alkyl of 1 to 17 carbon atoms, provided that $-L_{381}-$ and $-L_{382}-$ together have no more than 18 carbon atoms;

wherein $-F_{20}R_{10}-$ is $-NHR_{101}-$, $-NH(C=S)NHR_{102}-$ or $-NH(C=O)NHR_{102}-$;

wherein R_{101} is EDTAyl or DTPAyl and R_{102} is p-EDTA-phenyl or p-EDTA-benzyl; and

wherein R_{10} is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, provided that, if $F_{35}L_{35}F_{20}R_{10}$ is $-(CH_2)_2(CO)(NH)(CH_2)_kNHR_{101}$,

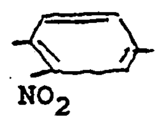
wherein k is 1 to 20, R_{101} is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

6. A nucleic acid probe according to Claim 1 which comprises:

(A) bonded to the 5'-terminal carbon, a group of formula $-OPO_2(NH)L_{10}F_{22}R_{221}$, $-OPO_3L_{11}SSL_{10}F_{20}R_{10}$, $-OPO_2S(CH_2)(CO)L_{10}F_{20}R_{10}$, or $-F_{20}R_{10}$; or

(B) bonded to the 3'-terminal carbon, if the 5'-terminal carbon is bonded to a phosphate group, an hydroxyl group or a group of formula $-OPO_2(NH)L_{10}F_{22}R_{221}$ or $-OPO_2S(CH_2)(CO)L_{10}F_{20}R_{10}$, a group of formula $-OPO_2(NH)L_{12}F_{23}R_{231}$ or $-OPO_2S(CH_2)(CO)L_{12}F_{21}R_{13}$, wherein L_{10} and L_{12} are the same or different and are each alkyl of 2 to 20 carbon atoms or a group of formula $-L_{201}(NH)(CO)L_{202}-$ or $-L_{201}(CO)(NH)L_{202}-$, wherein $-L_{201}$ is alkyl of 2 to 17 carbon atoms and wherein $-L_{202}-$ is alkyl of 1 to 17 carbon atoms and is bonded to $-F_{20}$, $-F_{21}$, $-F_{22}$ or $-F_{23}$ provided that L_{201} and L_{202} together have no more than 18 carbon atoms; wherein $-L_{11}-$ is alkyl of 3 to 20 carbon atoms; wherein the group $-F_{22}R_{221}$ or $-F_{20}R_{10}$ linked to the 5'-terminal carbon is the same as or different from the group $-F_{23}R_{231}$ or $-F_{21}R_{13}$ linked to the 3'-terminal carbon; wherein $-F_{20}R_{10}$ and $-F_{21}R_{13}$ are each selected from $-NHR_{11}$ or $-NH(C{=}R_{21})NHR_{12}$, wherein R_{11} is EDTAyl or DTPAyl, R_{12} is p-EDTA-phenyl or p-EDTA-benzyl, and R_{21} is oxygen or sulfur; wherein $-F_{22}R_{221}$ and $-F_{23}R_{231}$ are each selected from the group consisting of $-NHR_{11}$, $-NH(C{=}R_{21})R_{12}$, $-S(C{=}R_{21})R_{12}$ and $-S(CH_2)(CO)NHR_{12}$; and wherein $-R_{11}$ and $-R_{12}$ are optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}; provided that, if the 5'-terminal carbon is bonded to a group of formula $-OPO_2(NH)L_{10}NHR_{11}$, R_{11} is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

7. A nucleic acid probe made by a process which comprises reacting, with the nucleic acid with the sequence of the probe, (a) 1-(p-diazo-phenyl)EDTA which is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3} or (b) phenyl azide-derivatized DTPA or EDTA of formula $(R_{263})(NH)(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}NH(R_{261})$, wherein R_{261} is DTPAyl or EDTAyl, which is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, R_{263} is



$-N_3$, R_{264} is hydrogen or n-alkyl of 1 to 3 carbon atoms, aa is 1 to 6, bb is 1 to 6 and cc is 0 or 1.

8. A nucleic acid probe according to Claim 5 which is 12 to 100 nucleotides in length and which comprises a guanine or adenine moiety bonded through carbon-8 to a group of formula $-NH(CH_2)_cF_{20}R_{10}$, wherein c is 2 to 8, $-F_{20}R_{10}$ is $-NHR_{11}$ or $-NH(C{=}R_{21})NHR_{12}$, wherein R_{11} is EDTAyl or DTPAyl, R_{12} is p-EDTA-phenyl, R_{21} is O or S, and R_{10} is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

9. A probe according to Claim 8 wherein $-F_{20}R_{10}$ is $-NHR_{11}$.

10. A probe according to Claim 9 wherein R_{11} is complexed with Eu^{+3}.

11. A nucleic acid probe according to Claim 5 which comprises a cytosine moiety bonded through the N^4-nitrogen to a group of formula $-N{=}CH(CH_2)_dF_{20}R_{10}$, wherein d is 2 to 8, $F_{20}R_{10}$ is $-NHR_{11}$ or $-NH(C{=}R_{21})NHR_{12}$, wherein R_{11} is EDTAyl or DTPAyl, R_{12} is p-EDTA-phenyl, and R_{21} is O or S, and wherein R_{10} is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

12. A probe according to Claim 11 wherein $-F_{20}R_{10}$ is $-NHR_{11}$.

13. A probe according to Claim 12 wherein R_{11} is complexed with Eu^{+3}.

14. A probe according to Claim 13 which has at 12 to 100 nucleotides.

15. A nucleic acid probe according to Claim 5 comprising a uracil moiety or a cytosine moiety bonded at the 5-carbon to a group of formula $-(CH_2)_2(CO)(NH)L_{10}F_{20}R_{10}$ or $-CH{=}CH(CH_2)NH[(CO)L_{10}F_{20}]_eR_{10}$, wherein e is 0 or 1; wherein L_{10} is n-alkyl of 2 to 8 carbon atoms; wherein $-F_{20}-$ is -HN- or $-NH(C{=}R_{21})NH-$, wherein R_{21} is oxygen or sulfur; wherein, if $-F_{20}-$ is -NH-, or e is 0, R_{10} is EDTAyl or DTPAyl or, if $-F_{20}$ is $-NH(C{=}R_{21})NH-$, R_{10} is p-EDTA-phenyl, and wherein R_{10} is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, provided that, if the group bonded to the carbon-5 is of formula $-(CH_2)_2(CO)(NH)L_{10}NHR_{10}$, R_{10} is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

16. A probe according to Claim 15 wherein $-F_{20}-$ is -NH-.

17. A probe according to Claim 16 which is a DNA 12 to 10,000 nucleotides in length.

18. A probe according to Claim 17 which comprises a uracil moiety bonded to carbon-5 to a group of formula $-CH{=}CHCH_2NHR_{10}$.

19. A probe according to Claim 18 wherein the EDTAyl or DTPAyl is complexed with Eu^{+3}.

20. A probe according to Claim 6 wherein the group bonded to one or both of the 5'-terminal carbon and the 3'-terminal carbon is of formula $-OPO_2(NH)(CH_2)_f(NH)R_{11}$, wherein f is 2 to 20 and R_{11} is EDTAyl or DTPAyl.

21. A probe according to Claim 20 wherein f is to 2 to 8.

22. A probe according to Claim 21 wherein the EDTAyl or DTPAyl is complexed with Eu^{+3}.

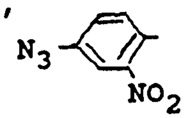
23. A probe according to Claim 22 which is 12 to 100 nucleotides in length and wherein the EDTAyl or DTPAyl is linked to only the 5'-terminal carbon.

24. A probe according to Claim 7 wherein the nucleic acid employed in the reaction is single-stranded.

25. A probe according to Claim 24 wherein the reaction is carried out with PDP-EDTA at a pH between about 7.5 and about 8.5, at a temperature between about 0°C and about 10°C, and with an initial molar concentration of 1-(p-diazo-phenyl) EDTA that is between about 0.1 times and about 2 times the molar concentration of nucleotides in the nucleic acid employed in the reaction, provided that such reaction is continued until, on the average, one nucleotide in 500 to one nucleotide in 50 in the reaction mixture is covalently linked to p-EDTA-phenyl.

26. A probe according to Claim 25 wherein the 1-(p-diazo-phenyl)EDTA employed in the reaction is complexed with Eu^{+3}.

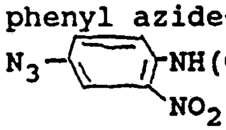
27. A probe according to Claim 26 wherein the nucleic acid employed in the reaction is a DNA and is 400 to 10,000 nucleotides in length.

28. A probe according to Claim 24 wherein the reaction is carried out with a phenyl azide-derivatized DTPA or EDTA of formula $(R_{263})(NH)(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}(NH)R_{261}$, wherein R_{261} is DTPAyl or EDTAyl, R_{263} is  ,

R_{264} is hydrogen or alkyl of 1 to 3 carbon atoms, aa is 1 to 6, bb is 1 to 6 and cc is 0 or 1, at a pH between about 6 and about 8 at a temperature between about 0°C and about 10°C, under illumination with light of wavelength between about 340 nm and 380 nm, and with an initial molar concentration of the phenyl azide-derivatized DTPA or EDTA that is between about 0.1 times and about 2 times the molar concentration of nucleotides in the nucleic acid employed in the reaction, provided that such reaction is continued until, on the average, one nucleotide in 500 to one nucleotide in 50 in the reaction mixture is covalently linked to the group R_{261}.

29. A probe according to Claim 28 wherein the nucleic acid employed in the reaction is a DNA and is 400 to 10,000 nucleotides in length.

30. A probe according to Claim 29 wherein the phenyl azide-derivatized compound is of formula

$NH(CH_2)_3(NCH_3)(CH_2)_3NH(DTPAyl)$.

31. A probe according to Claim 30 wherein the group R_{261} of the phenyl azide-derivatized compound employed in the reaction is complexed with Eu^{+3}.

32. A method of testing a sample for the presence of a biological entity, associated with a target DNA or RNA, which comprises:

(I) combining single-stranded nucleic acid of the sample with a nucleic acid probe for the target

DNA or RNA, said probe comprising a tag moiety wherein Eu^{+3}, Tb^{+3} or Sm^{+3} is chelated by EDTAyl DTPAyl, p-EDTA-phenyl or p-EDTA-benzyl, provided that the derivation of single-stranded nucleic acid from said sample and the combining of said single-stranded nucleic acid with said probe are carried out under conditions whereby stable duplexes form between probe and at least a portion of the target DNA or RNA present in said sample but not significanly between probe and non-target DNA or RNA; and

(II) determining whether stable duplex was formed in step (I) by

(A) separating unduplexed probe from duplexed probe formed in step (I);

(B) treating the product of step (I), after the separation of step (II)(A), to produce a fluorescent signal characteristic of the Eu^{+3}, Tb^{+3} or Sm^{+3} associated with any of the tag moiety that is present; and

(C) determining whether a detectable signal is generated by the treatment of step (II)(B).

33. A method according to Claim 32 wherein, after separation of duplexed from unduplexed probe and prior to fluorometry, an aqueous micelle suspension is formed, wherein the micelles include chelate with lanthanide ion dissociated from tag moiety of the probe that duplexed to target DNA or RNA, by combining probe that had duplexed with target DNA or RNA with an aqueous solution which is buffered to a pH between about 2.5 and about 4.5 and comprises (i) a non-ionic detergent; (ii) a synergistic base selected from O-phenanthroline, triphenylphosphine oxide or a trialkylphosphine oxide, wherein the alkyl groups are the same or different and are each alkyl of 1 to 10 carbon atoms; and (iii) a ß-diketone of formula $R_{51}(CO)CH_2(CO)CF_3$, wherein R_{51} is selected from 2-naphthyl, 1-naphthyl, 4-fluorophenyl, 4-methoxyphenyl, and phenyl.

34. A method according to Claim 33 wherein the nucleic acid probe comprises:

(A) a uracil or cytosine moiety bonded through carbon-5 to a group of formula $-F_{35}L_{35}F_{20}R_{10}$,

(B) a cytosine moiety bonded through the N^4-nitrogen to a group of formula $-F_{36}L_{36}F_{20}R_{10}$,

(C) a guanine or adenine moiety bonded through carbon-8 to a group of formula $-F_{38}L_{38}F_{20}R_{10}$;

wherein $-F_{35}-$ is -CH=CH-, -CH=CH(CO)(NH)-, $-CH{=}CHCH_2NH(CO)_a-$ or $-(CH_2)_2(CO)(NH)-$; wherein -CH= is bonded to carbon-5 and wherein a is 0 or 1; wherein, when $F_{35}-$ is -CH=CH-, -CH=CH(CO)(NH)-, $-(CH_2)_2(CO)(NH)-$ or a group terminated with a carbonyl group, L_{35} is n-alkyl of 1 to 20 carbon atoms or $-L_{351}(NH)(CO)L_{352}-$ or $-L_{351}(CO)(NH)L_{352}-$, wherein $-L_{351}-$ is n-alkyl of 1 to 17 carbon atoms and is bonded to $-F_{35}-$ and wherein $-L_{352}-$ is alkyl of 1 to 17 carbon atoms, provided that $-L_{351}-$ and $-L_{352}-$ together have no more than 18 carbon atoms; wherein, when $-F_{35}-$ is terminated with an amino group, L_{35} is $-CH_2(CHOH)CH_2O(CH_2)_bOCH_2(CHOH)CH_2-$, wherein b is 2 to 20;

wherein $-F_{36}-$ is -NH-, -NH(C=S)NH-, -NH(C=O)NH-, or $-N{=}C(R_{33})-$, wherein the nitrogen is bonded to the N^4-nitrogen and R_{33} is hydrogen or alkyl of 1 to 4 carbon atoms;

wherein $-L_{36}-$ is alkyl of 2 to 20 carbon atoms;

wherein $-F_{38}-$ is O, S or -NH-;

wherein $-L_{38}-$ is n-alkyl of 2 to 20 carbon atoms, $-L_{381}(NH)(CO)L_{382}-$ or $-L_{381}(CO)(NH)L_{382}-$, wherein $-L_{381}-$ is n-alkyl of 1 to 17 carbon atoms and is bonded to $-F_{38}-$ and $-L_{382}-$ is alkyl of 1 to 17 carbon atoms, provided that $-L_{381}-$ and $-L_{382}-$ together have no more than 18 carbon atoms;

wherein $-F_{20}R_{10}-$ is $-NHR_{101}-$, $-NH(C{=}S)NHR_{102}-$ or $-NH(C{=}O)NHR_{102}-$;

wherein R_{101} is EDTAyl or DTPAyl and R_{102} is p-EDTA-phenyl or p-EDTA-benzyl;

provided that R_{10} is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

35. A method according to Claim 34 wherein the nucleic acid probe

(A) has 12 to 100 nucleotides and comprises a guanine or adenine moiety bonded through carbon-8 to a group of formula $-NH(CH_2)_iNHR_{11}$, wherein i is 2 to 20; or

(B) has 12 to 10,000 nucleotides and comprises a uracil or cytosine moiety bonded through carbon-5 to a group of formula $-CH{=}CHCH_2(NH)R_{11}$; and

wherein R_{11} is EDTAyl or DTPAyl complexed with Eu^{+3}.

36. A method according to Claim 35 wherein, in the aqueous solution, the pH is buffered to between 3 and 4, the non-ionic detergent is 0.08 to 0.15% (v/v) Triton X-100, the synergistic base is tri-n-octylphosphine oxide and is present at 50 uM to 100 uM, and R_{51} of the ß-diketone is 2-naphythyl, 1-naphthyl or 4-fluorophenyl and the ß-diketone is present at 5 uM to 25 uM.

37. A method according to Claim 36 wherein the treatment to produce a fluorescent signal and determination of whether a detectable signal is generated comprise time-resolved fluorometry.

38. A method according to Claim 33 wherein the nucleic acid probe comprises:

(A) bonded to the 5'-terminal carbon, a group of formula $-OPO_2(NH)L_{10}F_{22}R_{221}$, $-OPO_3L_{11}SSL_{10}F_{20}R_{10}$, $-OPO_2S(CH_2)(CO)L_{10}F_{20}R_{10}$, or $-F_{20}R_{10}$; or

(B) bonded to the 3′-terminal carbon, if the 5′-terminal carbon is bonded to a phosphate group, an hydroxyl group or a group of formula $-OPO_2(NH)L_{10}F_{22}R_{221}$ or $-OPO_2S(CH_2)(CO)L_{10}F_{20}R_{10}$, a group of formula $-OPO_2(NH)L_{12}F_{23}R_{231}$ or $-OPO_2S(CH_2)(CO)L_{12}F_{21}R_{13}$, wherein L_{10} and L_{12} are the same or different and are each alkyl of 2 to 20 carbon atoms or a group of formula $-L_{201}(NH)(CO)L_{202}-$ or $-L_{201}(CO)(NH)L_{202}-$, wherein $-L_{201}$ is alkyl of 2 to 17 carbon atoms and wherein $-L_{202}-$ is alkyl of 1 to 17 carbon atoms and is bonded to $-F_{20}$, $-F_{21}$, $-F_{22}$ or $-F_{23}$ provided that L_{201} and L_{202} together have no more than 18 carbon atoms; wherein $-L_{11}-$ is alkyl of 3 to 20 carbon atoms; wherein the group $-F_{22}R_{221}$ or $-F_{20}R_{10}$ linked to the 5′-terminal carbon is the same as or different from the group $-F_{23}R_{231}$ or $-F_{21}R_{13}$ linked to the 3′-terminal carbon; wherein $-F_{20}R_{10}$ and $-F_{21}R_{13}$ are each selected from $-NHR_{11}$ or $-NH(C{=}R_{21})NHR_{12}$, wherein R_{11} is EDTAyl or DTPAyl, R_{12} is p-EDTA-phenyl or p-EDTA-benzyl, and R_{21} is oxygen or sulfur; wherein $-F_{22}R_{221}$ and $-F_{23}R_{231}$ are each selected from the group consisting of $-NHR_{11}$, $-NH(C{=}R_{21})R_{12}$, $-S(C{=}R_{21})R_{12}$ and $-S(CH_2)(CO)NHR_{12}$; and wherein $-R_{11}$ and $-R_{12}$ are optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}; provided that, if the 5′-terminal carbon is bonded to a group of formula $-OPO_2(NH)L_{10}NHR_{11}$, R_{11} is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

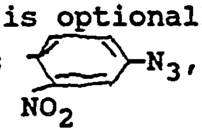
39. A method according to Claim 38 wherein the probe has 12 to 100 nucleotides and wherein the group bonded to one or both of the 5′-terminal carbon and the 3′-terminal carbon is of formula $-OPO_2(NH)(CH_2)_jNHR_{11}$, wherein j is 2 to 8.

40. A method according to Claim 39 wherein the EDTAyl or DTPAyl is linked to only the 5'-terminal carbon and is complexed with Eu^{+3}.

41. A method according to Claim 40 wherein, in the aqueous solution, the pH is buffered to between 3 and 4, the non-ionic detergent is 0.08 to 0.15% (v/v) Triton X-100, the synergistic base is tri-n-octylphosphine oxide and is present at 50 uM to 100 uM, and R_{51} of the ß-diketone is 2-naphythyl, 1-naphthyl or 4-fluorophenyl and the ß-diketone is present at 5 uM to 25 uM.

42. A method according to Claim 41 wherein the treatment to produce a fluorescent signal and determination of whether a detectable signal is generated comprise time-resolved fluorometry.

43. A method according to Claim 33 wherein the nucleic acid probe is a probe made by a process comprising

(A) reacting, with the nucleic acid with the sequence of the probe, (a) 1-(p-diazo-phenyl)EDTA which is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3} or, (b) under photoactivating conditions, a phenyl azide-derivatized compound of formula $(R_{263})(NH)(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}NH(R_{261})$, wherein R_{261} is DTPAyl or EDTAyl, which is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, R_{263} is ,

R_{264} is hydrogen or n-alkyl of 1 to 3 carbon atoms, aa is 1 to 6, bb is 1 to 6 and cc is 0 or 1; and,

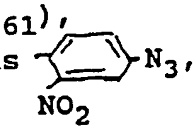
(B) if the 1-(p-diazo-phenyl)EDTA or phenyl azide-derivatized compound employed in step (A) to make the probe is not complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, subjecting the probe from said step to the standard probe chelation process with a salt of Eu^{+3}, Sm^{+3} or Tb^{+3}.

44. A method according to Claim 43 wherein the reaction whereby the probe is made is carried out with 1-(p-diazo-phenyl)EDTA on single-stranded nucleic acid and at a pH between about 7.5 and about 8.5, at a temperature between about 0°C and about 10°C, and with an initial molar concentration of 1-(p-diazo-phenyl) EDTA that is between about 0.1 times and 2 times the molar concentration of nucleotides in the nucleic acid employed in the reaction, provided that such reaction is continued until, on the average, between about one nucleotide in 50 and about one nucleotide in 500 in the reaction mixture is covalently bonded to p-EDTA-phenyl and using 1-(p-diazo-phenyl)EDTA which is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

45. A method according to Claim 44 wherein the p-EDTA-phenyl label of the probe is complexed with Eu^{+3} and wherein the reaction whereby the probe is made is carried out on a single-stranded nucleic acid of 400 to 10,000 bases in length.

46. A method according to Claim 45 wherein, in the aqueous solution that is combined with probe that had duplexed with target DNA or RNA, the pH is buffered to between 3 and 4, the non-ionic detergent is 0.08 to 0.15% (v/v) Triton X-100, the synergistic base is tri-n-octylphosphine oxide and is present at 50 uM to 100 uM, and R_{51} of the ß-diketone is 2-naphthyl, 1-naphthyl or 4-fluorophenyl and the ß-diketone is present at 5 uM to 25 uM.

47. A method according to Claim 46 wherein the treatment to produce a fluorescent signal and determination of whether a detectable signal is generated comprise time-resolved fluorometry.

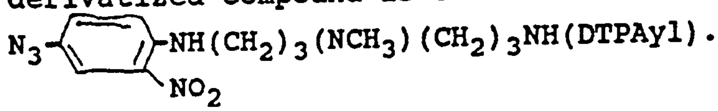
48. A method according to Claim 43 wherein the reaction whereby the probe is made is carried out with a phenyl azide-derivatized compound of formula $(R_{263})(NH)(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}(NH)(R_{261})$, wherein R_{261} is DTPAyl or EDTAyl, R_{263} is , R_{264} is hydrogen or n-alkyl of 1 to 3 carbon atoms, aa is 1 to 6, bb is 1 to 6 and cc is 0 or 1, on single-stranded nucleic acid and at a pH between about 6 and about 8 at a temperature between about 0°C and about 10°C, under illumination with light of wavelengths between about 340 nm and 380 nm, and with an initial molar concentration of the phenyl azide derivatized compound that is between about 0.1 times and 2 times the molar concentration of nucleotides in the nucleic acid employed in the reaction, provided that such reaction is continued until, on the average, between about one nucleotide in 50 and about one nucleotide in 500 in the reaction mixture is covalently linked to the group R_{261} and using in step (A) phenyl azide derivatized compound which is complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

49. A method according to Claim 48 wherein the reaction whereby the probe is made is carried out on a single stranded nucleic acid of 400 to 10,000 bases in length.

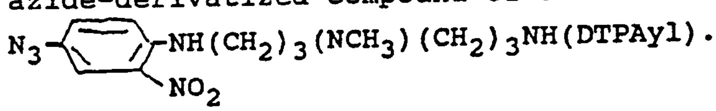
50. A method according to Claim 40 wherein, in the aqueous solution that is combined with probe that had duplexed with target DNA or RNA, the pH is buffered to between 3 and 4, the non-ionic detergent is 0.08 to 0.15% (v/v) Triton X-100, the synergistic base is tri-n-octylphosphine oxide and is present at 50 uM to 100 uM, and R_{51} of the ß-diketone is 2-naphthyl, 1-naphthyl or 4-fluorophenyl and the ß-diketone is present at 5 uM to 25 uM.

51. A method according to Claim 50 wherein the treatment to produce a fluorescent signal and determination of whether a detectable signal is generated comprise time-resolved fluorometry.

52. A method according to Claim 50 wherein, in the reaction whereby the probe is made, the phenyl azide derivatized compound is of formula



53. A method according to Claim 51 wherein, in the reaction whereby the probe is made, the phenyl azide-derivatized compound of formula



54. A nucleic acid probe with a sequence selected from the single-stranded DNA sequences:

5'-AACCAACAAGAAGATGAGGCATAGCAGCA-3' and

5'-TGCTGCTATGCCTCATCTTCTTGTTGGTT-3' and the single-stranded RNA sequences:

5'-AACCAACAAGAAGAUGAGGCAUAGCAGCA-3' and

5'-UGCUGCUAUGCCUCAUCUUCUUGUUGGUU-3'.

55. A nucleic acid which is a single-stranded DNA of sequence

5'-AACCAACAAGAAGATGAGGCATAGCAGCA-3' or

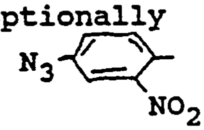
5'-TGCTGCTATGCCTCATCTTCTTGTTGGTT-3' or a single-stranded RNA sequence:

5'-AACCAACAAGAAGAUGAGGCAUAGCAGCA-3' or

5'-UGCUGCUAUGCCUCAUCUUCUUGUUGGUU-3' and wherein the 5'-terminal carbon and 3'-terminal carbon are bonded to moieties, other than hydrogen and neighboring carbons, selected from the entries in Table XLV:

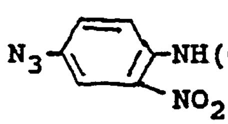
TABLE XLV

Moiety bonded to 5′-Terminal Carbon	Moiety bonded to 3′-Terminal Carbon
$-OH$	$-OH$
$-OPO_3$	$-OH$
$-NH_2$	$-OH$
$-O\overset{S}{\overset{\|}{P}}O_2$	$-OH$
$-OPO_3(CH_2)_jSH$, wherein j is 3 to 8	$-OH$
$-OH$	$-OPO_3$
$-OPO_3$	$-OPO_3$

56. A compound of formula $(R_{263})(NH)(CH_2)_{aa}(NR_{264})_{cc}(CH_2)_{bb}(NH)(R_{261})$, wherein R_{261} is DTPAyl or EDTAyl, which is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}, R_{263} is 

R_{264} is hydrogen or n-alkyl of 1 to 3 carbon atoms, aa is 1 to 6, bb is 1 to 6 and cc is 0 or 1.

57. A compound according to Claim 56 of formula

$-NH(CH_2)_3(NCH_3)(CH_2)_3NH(DTPAyl)$,

wherein the DTPAyl is optionally complexed with Eu^{+3}, Tb^{+3} or Sm^{+3}.

58. The compound according to Claim 57 wherein the DTPAyl is complexed with Eu^{+3}.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/03735

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4th Ed.): C12Q 1/68; C07H 19/207; C07C 117/00, 121/86

US Cl.: 435/6; 536/27-29; 260/349; 436/800; 436/805; 534/14

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/6; 536/27-29; 260/349; 436/800; 436/805; 534/14

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Computer Sequence Search: Quest-NIH

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	EP, B, 0 154 788, ENZO BIOCHEM, INC., Published 18 September 1985 , see pp. 1-81.	1-58
P,Y	US, A, 4,711,955, WARD ET AL, Published 08 December 1987, see coumns 1-34.	1-58
Y	Clinical Chemistry, Volume 29, Issued January 1983, E. Soini et al, "Time-Resolved Fluorometer for Lanthanide Chelates - A New Generation of Nonisotopic Immuno-assays." See pp. 65-68.	1-58
Y	Analytical Biochemistry, Volume 137, Issued 1984, (New York, New York), I. Hemmila et al, "Europium as a Label in Time-Resolved Immunofluorometric Assays.", see pp. 335-343.	1-58
Y	US, A, 4,565,790, (HEMMILA ET AL) 21 January 1986, See column 2.	1-58
Y	Proceeding of the National Academy of Science U.S.A., Volume 82, Issued	1-58

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
17 February 1989	07 APR 1989
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	L. Eric Crane

International Application No. PCT/US88/03735

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

	February 1985, (Washington, D.C.) B.C.F. Chu et al, "Nonenzymatic sequence-specific cleavage of single-stranded DNA.", see pp. 963-967.	
Y	Chemical Reviews, Volume 82, Issued 1982, (Easton, PA), F. S. Richardson, "Terbium (III) and Europium (III) Ions as Luminescent Probes and Stains for Biomolecular Systems", See pp. 541-552.	1-58
Y	US, A, 4,374,120, (SOINI ET AL) 15 February 1983, See columns 1-6.	1-58

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers ......, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

International Application No. PCT/US88/03735

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	US, A, 4,563,419, (RANKI ET AL) 07 January 1986, see columns 1-16	1-58
Y	Nature, Volume 281, Issued 25 October 1979, (Great Britian), F. Galibert et al, "Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E coli.", see pp. 646-649.	1-58